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REGULATION OF BLOOD OXYGEN AFFINITY IN THE AUSTRALIAN BLACKFISH *GADOPSIS MARMORATUS*

I. CORRELATIONS BETWEEN OXYGEN-BINDING PROPERTIES, HABITAT AND SWIMMING BEHAVIOUR

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SUMMARY

1. The regulation of whole blood oxygen affinity in the freshwater blackfish *Gadopsis marmoratus* Richardson has been examined, and correlations made between oxygen-binding properties and the habitat and swimming behaviour of the fish.

2. Blackfish whole blood has a low oxygen affinity relative to other fish bloods reported in the literature. This is not due to a low oxygen affinity of the stripped haemoglobins, but arises from interactions between haemoglobin and intraerythrocytic modulators.

3. The presence of high concentrations of ATP, and to a lesser extent GTP, in the erythrocyte, together with the effect of these nucleoside triphosphates on the oxygen affinity of haemoglobin solutions at physiological NTP: Hb_4 molar ratios, demonstrates that this class of compounds is a major regulator of oxygen affinity in blackfish blood.

4. The oxygen affinities of whole blood and haemoglobin solutions are sensitive to pH, with haemoglobin solutions displaying a relatively large alkaline Bohr coefficient of $-1{\cdot}05$ over the physiologically relevant pH range of 6·5–7·0.

5. Although increasing P_{CO_2} lowers the oxygen affinity of whole blood, it does so only through the effect on pH, as pH-buffered haemoglobin solutions show no oxygen-linked CO_2 binding. This lack of oxygen-linked CO_2 binding has not been reported for any other naturally occurring vertebrate haemoglobins.

6. Muscle morphology and biochemistry, and behavioural observations, indicate that the blackfish uses anaerobic energy metabolism during rapid swimming and in recovery.

7. It is concluded that the oxygen-binding properties of blackfish blood reflect adaptations for maintaining adequate tissue oxygenation for animals at rest and during slow sustained swimming in waters of high oxygen tensions.

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INTRODUCTION

The oxygen equilibrium curve of whole blood is an important component of the oxygen transport system in water-breathing fish. The shape and position of the curve represents a compromise between the need to obtain sufficient oxygen from an environment that, relative to air, is oxygen deficient, and the need to permit satisfactory unloading at the tensions encountered in tissue capillaries. Thus it is not surprising to find that species-specific differences in the oxygen equilibrium curve reflect differences in environmental oxygen availability and in behaviour and physiology (Krogh & Leitch, 1919; Willmer, 1934; Wood & Lenfant, 1979). This 'fine' tuning of the oxygen equilibrium curve involves both the intrinsic oxygen-binding properties of haemoglobin, and highly specific interactions between haemoglobin and a number of allosteric modulators operating within the erythrocyte. Among the most important of these modulators of haemoglobin function are organic phosphates, pH, CO_2 and certain inorganic ions (Benesch & Benesch, 1967; Chanutin & Curnish, 1967).

The individual effects of these modulators have been well documented for fish haemoglobins (for review see Riggs, 1979; Wood, 1980), but the aim of the present study was to investigate the relative importance of each modulator in adjusting the oxygen equilibrium curve of whole blood in the freshwater blackfish *Gadopsis marmoratus*, and seek correlations between the effects of these modulators and the habitat and swimming behaviour of this fish.

The influence of environmental temperature, another important determinant of haemoglobin function in water-breathing fish, is considered in an accompanying paper (Dobson & Baldwin, 1982).

MATERIALS AND METHODS

Blackfish (*Gadopsis marmoratus*) were collected by electrofishing in the Yarra river, 20 km east of Warburton, Victoria, Australia. Mature fish ranging in length from 18 to 25 cm were caught during the summer months when the water temperatures averaged 20 °C. The animals were maintained for at least a month at 20 °C in filtered aerated aquaria housed in a constant-temperature room set on a 12 h light/12 h dark cycle. During this period fish were fed *ad libitum* on chopped liver.

Fish were stunned by a blow to the head and the tail severed behind the caudal fin. Blood was collected by inserting the needle of a heparinized syringe directly into the caudal vessels, thereby reducing contamination from other body fluids. A limitation of this sampling technique is that measured blood pH may not represent true resting *in vivo* values; however, to minimize such changes fish were captured, stunned and bled within 45 s.

Haemolysates were prepared by centrifuging fresh whole blood for 5 min at 3000 *g* and 4 °C. The packed erythrocytes were washed 3 times with ice-cold 150 mM-NaCl. Bis-tris or tris-HCl buffers were added to the packed cells to give a final molarity of 50 mM. The suspension was sonicated at 0 °C followed by centrifugation for 10 min at 4000 *g* and 4 °C. Small molecules such as organic phosphates were stripped from the haemolysate solution using the ultrafiltration method of Jelkman & Baeur (1976).

Haematocrits were determined by the micro-method using an IEC model MB micro-capillary centrifuge. Haemoglobin concentration was determined by the spectrophotometric method of Van Kampen & Zijlstra (1965). Methaemoglobin was determined by the method of Evelyn & Malloy (1938). The oxygen-carrying capacity of whole blood was calculated using the method of Dijkhuizen *et al.* (1977).

The concentrations of total nucleoside triphosphates (NTP), and 2,3-diphosphoglycerate (2,3-DPG) were determined enzymically using test kits (Boehringer Mannheim). Specific nucleotides were separated and quantitated by column chromatography as described by Isaaks *et al.* (1976) and Bartlett (1978). Column fractions were analysed by absorbance at 260 nm and assayed for total phosphate using the method of Ames & Dubin (1960).

pH measurements were made with a Radiometer blood pH electrode at 20 °C coupled to an acid–base analyser (Radiometer PHM Mk II). Intraerythrocytic pH values were determined using the method of Enoki *et al.* (1972). Similar values were obtained using the alternative procedure of Murphy *et al.* (1977).

Oxygen equilibrium curves of whole blood and concentrated haemoglobin solutions were determined at 20 °C by the mixing technique described by Edwards & Martin (1966) after Haab, Piiper & Rahn (1960). This technique involved mixing known volumes of fully oxygenated and fully deoxygenated blood in varying proportions using a specially calibrated 1·0 ml gas-tight syringe, then measuring the partial pressure of oxygen. Partial pressures of oxygen (P_{O_2}) were determined with a Radiometer oxygen electrode (E 5046) housed in a Radiometer thermostated cell (D 616). The electrode was coupled to a Radiometer acid–base analyser (PHM 71 Mk II). The method was verified using human blood as a standard at a P_{CO_2} of 40 mmHg and 38 °C. The whole curve was determined and the P_{50} value of 26·5 ± 0·5 and n value of 2·98 are in good agreement with the published data of Dill & Forbes (1941) and Astrup *et al.* (1965). Blood and haemoglobin solutions were equilibrated for 10–15 min in 25 ml temperature-controlled tonometers with humidified gas mixtures comprising 3·95 mmHg in air and in nitrogen. Gases were supplied and analysed by C.I.G., Victoria.

The Root effect of blackfish haemoglobin was determined by the spectrophotometric method described by Weber & De Wilde (1975).

The relative proportions of red and white muscle along the body of the blackfish were measured by the method of Mosse & Hudson (1977).

Maximum activities of the enzymes hexokinase, phosphorylase, phosphofructokinase and lactate dehydrogenase in blackfish white muscle were determined as described by Baldwin & Seymour (1977). The lactate dehydrogenase isoenzymes in muscle homogenates were separated by electrophoresis and quantified as described by Muller & Baldwin (1978). The subunit compositions of these isoenzymes were determined immunochemically using antisera prepared against the M_4 and H_4 lactate dehydrogenase enzymes purified from *Sardinops neopilchardus* (Holmes & Scopes, 1974).

The concentrations of lactate in blood and muscle were measured enzymically using test kits (Boehringer Mannheim). Muscle samples were prepared for analysis using standard procedures described in Bergmeyer (1974). Blood lactates were determined using 20% trichloroacetic acid extracts of whole blood (5:1 vol/vol).

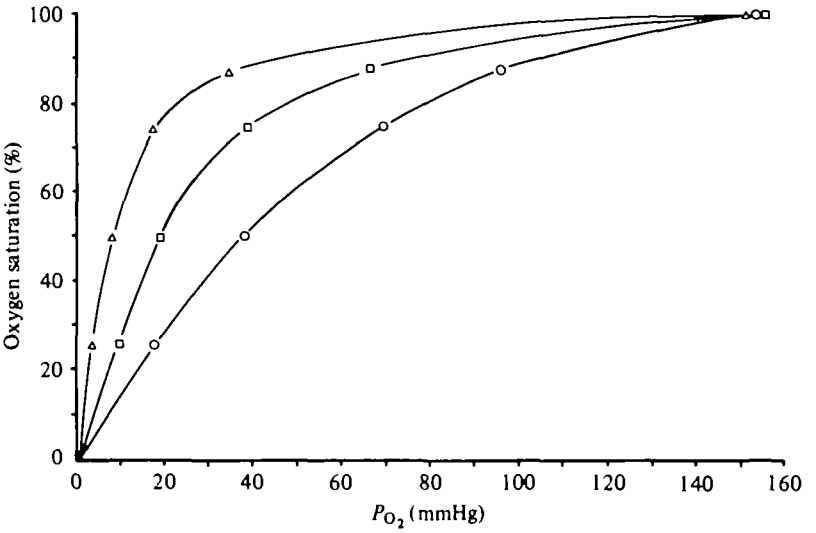

Fig. 1. Oxygen equilibrium curves for whole blood (○), sonicated whole blood (△), and stripped haemoglobin (□). Experimental conditions: assay temperature, 20 °C; equilibration P_{O_2}, 3·95 mmHg; whole blood pH, 7·52; sonicated whole blood pH, 7·49; stripped haemoglobin solution pH, 7·0; haemoglobin concentration of stripped solution, 2·0 g/100 ml buffered in 0·05 M-bis-tris HCl, 0·1 M-NaCl. Each point determination was done in triplicate with ±0·5–1·0 mmHg variability.

RESULTS

Respiratory properties of whole blood

The oxygen equilibrium curve for blackfish whole blood is plotted in Fig. 1, and the oxygen-binding properties are summarized in Table 1 together with published data for other freshwater fishes. The P_{50} values listed in Table 1 show that the blackfish whole blood curve is positioned to the right of the oxygen equilibrium curves reported for other fishes.

Intraerythrocytic modulation of haemoglobin oxygen affinity

The experimental system

Oxygen equilibrium curves for blackfish whole blood, sonicated whole blood (cells lysed and haemoglobin released into plasma) and a solution of stripped haemoglobin adjusted to the intraerythrocytic pH of 7·0, are shown in Fig. 1. Haemolysis has a marked effect on the position and shape of the whole blood curve, with the P_{50} decreasing from 37·5 to 7·0 mmHg, and Hill coefficient, n, decreasing from 1·58 to 1·20. The position of the whole blood oxygen equilibrium curve is not restored for a solution of stripped haemoglobin adjusted to intraerythrocytic pH value of 7·0 (P_{50} is 18·0 mmHg, n is 1·60).

Organic phosphates

Enzymic analysis of red cell extracts from blackfish showed high concentrations of nucleoside triphosphates (Table 3). The mean concentration was 6·43 μmole NTP/ml packed erythrocytes, giving a molar ratio of total NTP:Hb_4 of 1·68. The major

Table 1. *A comparison between the oxygen equilibrium curves of whole blood from a range of freshwater fish, differing in habitat preference and activity pattern*

Species	temp (°C) Assay	Assay P_{CO_2} (mmHg)	P_{50}*	n†	Shape of curve	Habitat preference	Activity pattern	Reference
1. High-affinity blood								
Cyprinus carpio (European carp)	17	1–2	2–3	1·30	Hyperbolic	Stagnant waters	Sluggish	Redfield (1933), Black (1940)
Ictalurus nebulosus (catfish)	24	0–1	7·3	1·23	Hyperbolic	Stagnant waters	Sluggish	Haws & Goodnight (1962), Grigg (1969, 1974)
Tinca tinca (tench	20	3·5	7·0	—	—	Slow still waters	Sluggish	Eddy (1973)
2. Low-affinity blood								
Salmo gairdneri (rainbow trout)	20	3	27	2·2	Sigmoidal	Well-oxygenated waters	Strong active swimmers	Cameron (1971)
Salvelinus fontanilis (brook trout)	22	1–2	26	2·8	Sigmoidal	Well-oxygenated waters	Strong active swimmers	Irving *et al.* (1941), Black *et al.* (1966)
Gadopsis marmoratus (river blackfish)	20	3·95	37·5	1·58	Hyperbolic	Well-oxygenated waters	Short-term swimmers	This study

* Partial pressure of oxygen at which half the haemoglobin is saturated with oxygen, expressed in mmHg.
† Hill coefficient, n, calculated between 25 % and 75 % HbO_2.

Table 2. *Some respiratory properties of whole blood obtained for resting blackfish maintained at* 20 °C

Whole blood pH	7·50 ± 0·08 (21)
Intra-erythrocytic pH	7·05 ± 0·03 (10)
Blood P_{CO_2}, mmHg	3·50 ± 0·50 (5)
Blood bicarbonate concentration, mM	3·66 ± 0·54 (5)
Haematocrit (%)	21·3 ± 2·23 (13)
Blood haemoglobin concentration (g/100 ml)	5·19 ± 0·33 (13)
Estimate of blood oxygen-carrying capacity (g/100 ml)	7·10 ± 0·45 (13)
Methaemoglobin concentration (% of total haemoglobin)	4·20 ± 2·45 (15)
Whole blood P_{50} at 3·95 mmHg CO_2 and pH 7·52 (mmHg)	37·5 ± 1·56 (21)
Hill coefficient, n	1·58

Values given are the mean ± standard deviation with the number of fish assayed in parentheses.

Table 3. *Concentrations of organic phosphate compounds in the erythrocytes of blackfish maintained at* 20 °C

Organic phosphate	μmole/ml rbc
Total NTP	6·43 ± 0·53 (13)
NTP:Hb_4 ratio	1·68 ± 0·18 (13)
ATP (total phosphate analysis)	5·49 (8)
ATP:Hb_4 ratio	1·34
GTP (total phosphate analysis)	1·34 (8)
GTP:Hb_4	0·34
≃ATP:GTP ratio	5:1
ATP + GTP ≃ total NTP (total phosphate analysis)	6·83
2,3-Diphosphoglycerate	< 0·05 (7)

Values given are the mean ± standard deviation with the number of fish assayed in parentheses.

organic phosphate in erythrocytes of most mammals, 2,3-DPG, was not detected. Ion-exchange chromatography revealed that the total NTP comprised ATP and GTP in the ratio 5:1. Total phosphate analysis showed that the ATP and GTP recovered from the column accounted for the total NTP measured in whole blood (Fig. 2, Table 3).

The effects of ATP and GTP on the oxygen affinity (P_{50}) of stripped haemoglobin solutions are shown in Fig. 3. Increasing concentrations of both ATP and GTP

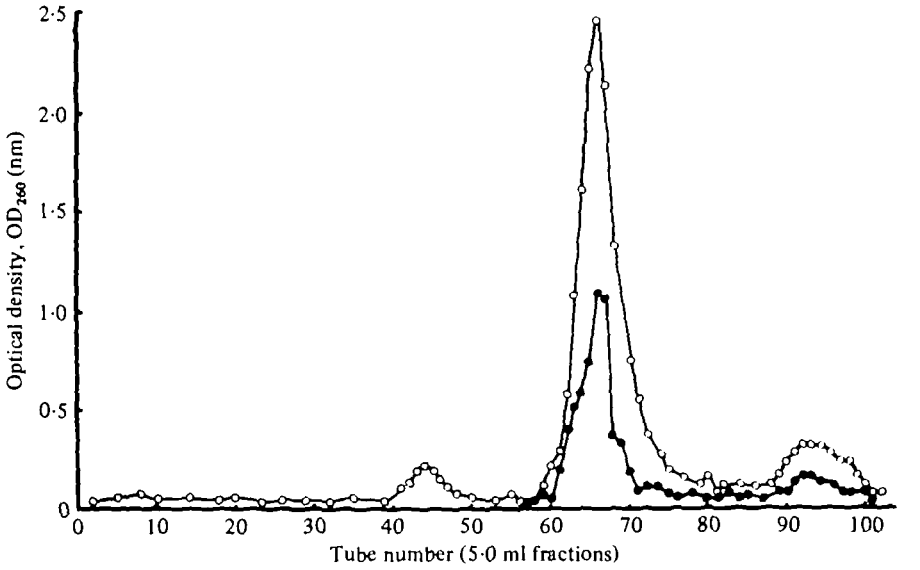

Fig. 2. Anion-exchange chromatography of acid-soluble organic phosphates in blackfish erythrocytes. A-260 nm, ○; Total phosphate, ●; peak at tube number 68, ATP; peak at tube number 93, GTP.

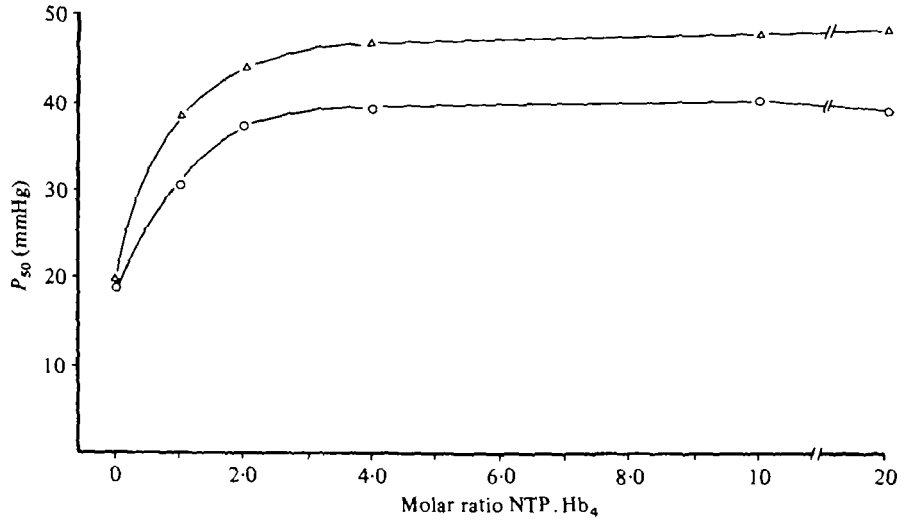

Fig. 3. Effects of nucleoside triphosphates on the oxygen affinity (P_{50}) of a solution of stripped haemoglobin. ATP, ○; GTP, △. Experimental conditions: assay temperature 20 °C; equilibration P_{O_2}, 0 mmHg; haemoglobin concentration, 1·04 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl, pH 7·0. The molar ratio of NTP:Hb_4 is defined as the molar ratio of ATP and GTP per haemoglobin tetramer. Each point was done in triplicate with ± 1·0 mmHg variability.

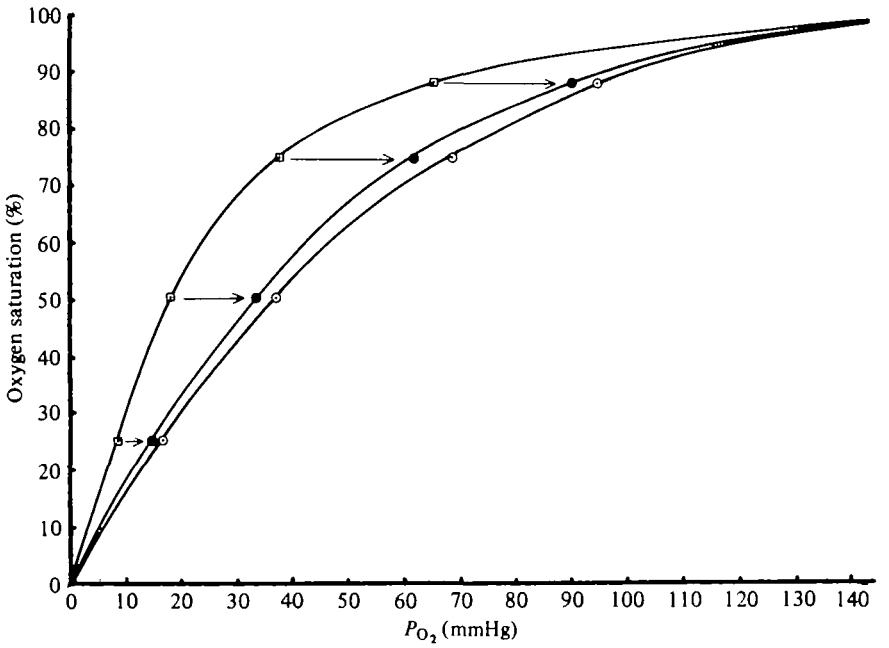

Fig. 4. Effect of a physiological molar ratio of ATP on the position of the oxygen equilibrium curve of stripped haemoglobin, compared with the oxygen equilibrium curve of whole blood: stripped haemoglobin solution, □; stripped haemoglobin solution with ATP:Hb_4 = 1·5 ●; whole blood, ○. Arrows indicate shift of the curve obtained for stripped haemoglobin following the addition of ATP. Experimental conditions: assay temperature, 20 °C; equilibrium P_{CO_2}, 3·95 mmHg; haemoglobin concentration of stripped solutions, 2·0 g/100 ml in 0·05 M-bis-tris HCl buffer, 0·1 M-NaCl, pH 7·0.

markedly decrease oxygen affinity (increase P_{50}), with the maximum decrease occurring at molar ratios between 1·0 and 2·0. For a given molar ratio, GTP is more effective in reducing the oxygen affinity of blackfish haemoglobin.

An *in vitro* system designed to mimic the whole blood curve at 20 °C was constructed by adding physiological levels of ATP to a solution of stripped haemoglobin at intraerythrocytic pH (Fig. 4). Following the addition of ATP, the oxygen affinity decreased from a P_{50} value of 18·0 to 34·0 mmHg, a value which is similar to that obtained for whole blood. The Hill coefficient, n, remained essentially unchanged at 1·60.

Carbon dioxide

The mean partial pressure of carbon dioxide in blood taken from the caudal vessels was 3·5 ± 0·5 (Table 2).

The effects of increasing the partial pressure of carbon dioxide (P_{CO_2}) on the oxygen affinity of whole blood, and haemoglobin solutions, are shown in Fig. 5. Increasing P_{CO_2} over the range 3·95 to 22·4 mmHg decreases the oxygen affinity of whole blood, with P_{50} values increasing from 17·0 to 65·0 mmHg. The CO_2–Bohr coefficient ($\Delta \log P_{50}/\Delta$ pH) over this range was −0·625 (Fig. 6). In marked contrast, the oxygen affinity of strongly pH-buffered haemoglobin solutions is insensitive to changes in

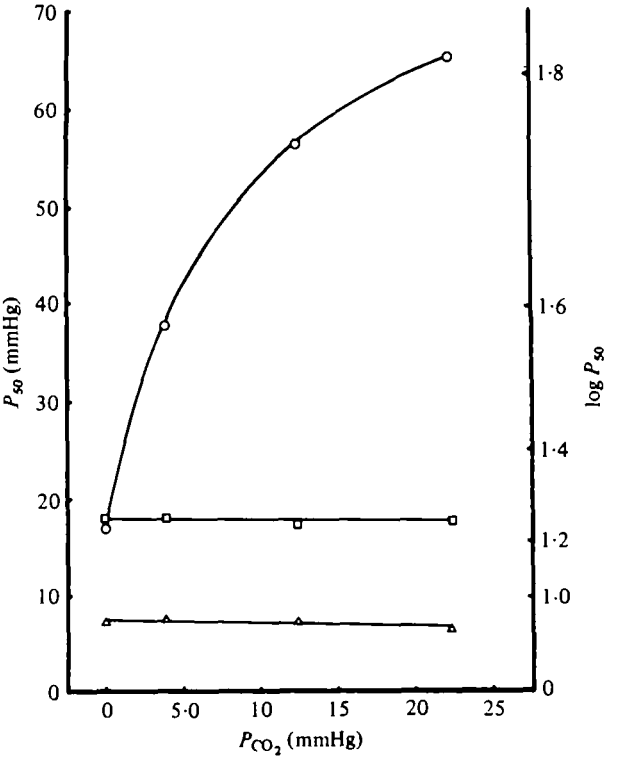

Fig. 5. Effect of CO_2 on the oxygen affinity (P_{50}) of whole blood and stripped haemoglobin solutions buffered at a constant pH of 7·0 and 7·4; whole blood, ○; haemoglobin solution at pH 7·0, □; haemoglobin solution at pH 7·4, △. Experimental conditions: assay temperature, 20 °C; equilibration P_{CO_2}, 0, 3·95, 12·4 and 22·4 mmHg; whole blood pH, 7·5 when introduced into tonometer; whole blood haemoglobin concentration, 5·5 g/100 ml; haemoglobin concentration of stripped solutions, 2·5 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl, pH 7·0 or 7·4. Each point for whole blood was done in duplicate with ±0·5–1·0 mmHg variability.

carbon dioxide tension, both in the presence and in the absence of 100 mM sodium chloride and ATP (Fig. 5, Table 4).

Hydrogen ion concentration

In 20 °C-acclimated blackfish, the mean intraerythrocytic pH of 7·05 is considerably lower than the value of 7·52 obtained for whole blood (Table 2). The effects of pH on oxygen affinity and n values for haemoglobin solutions in the presence and absence of ATP are shown in Figs. 6 and 7. Blackfish haemoglobin is very sensitive to pH over the range 6·5–7·0, with decreasing pH leading to a marked increase in P_{50}. The alkaline Bohr coefficient in this case is $-1{\cdot}05$ between pH 6·5 and 7·0, a range that probably represents intraerythrocytic values (see Table 2, Fig. 10). Accompanying this large decrease in oxygen affinity at low pH is an increase in Hill coefficient, n, which changes from 1·6 at pH 7·0 to 2·5 at pH 6·5, as the oxygen equilibrium curve becomes increasingly sigmoidal.

When blackfish were exercised to exhaustion for 1 min, whole blood pH fell, reach-

Table 4. *The effect of CO_2 on the oxygen affinity of haemoglobin with and without ATP* for 20 °C-acclimated blackfish at 20 °C*

	P_{50} at 0 mmHg CO_2		P_{50} at 3·95 mmHg CO_2		P_{50} at 12·4 mmHg CO_2	
1. Solution pH 7·0	(No NaCl)	(0·1 M-NaCl)	(No NaCl)	(0·1 M-NaCl)	(No NaCl)	(0·1 M-NaCl)
'Stripped' haemoglobin	18·0	18·0	18·0	17·5	18·0	18·0
'Stripped' haemoglobin + ATP (ATP: Hb_4 = 2·0)	17·5	18·0	18·0	18·0	17·5	18·0
2. Solution pH 7·4						
'Stripped' haemoglobin	7·0	7·0	6·5	7·0	7·0	6·5
'Stripped' haemoglobin + ATP (ATP: Hb_4 = 2·0)	7·0	7·0	7·0	6·5	7·0	7·0

* Adenosine-5-triphosphate.

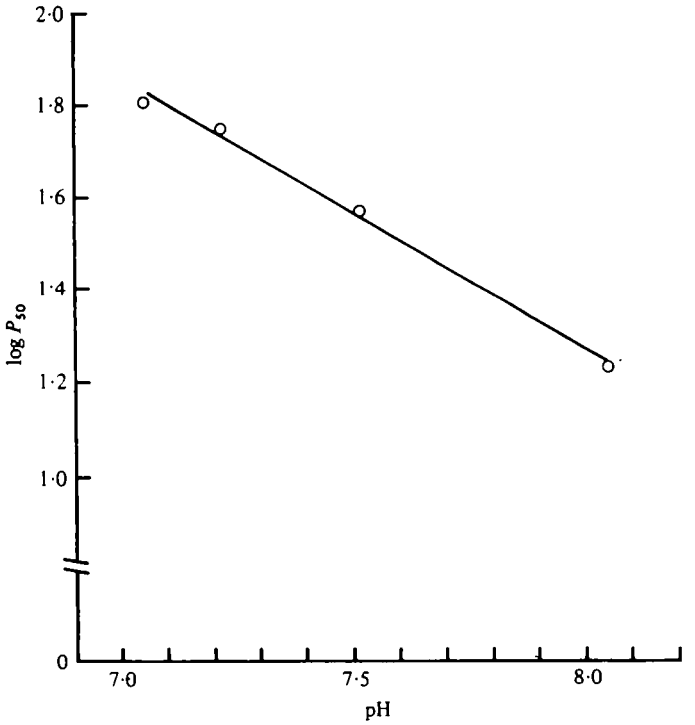

Fig. 6. CO_2–Bohr effect of whole blood. Data obtained from Fig. 5, except that log P_{50} is plotted against the pH of oxygenated whole blood measured at each equilibration P_{CO_2} at 20 °C. Bohr coefficient, ϕ, = $\Delta \log P_{50}/\Delta$pH
= −0·625 over the pH range 7·05–8·05.

ing a minimum of 7·17 after 30 min recovery (Fig. 10). The CO_2–Bohr effect for whole blood (Fig. 5) shows that this decrease in blood pH would result in a 1·5-fold reduction in oxygen affinity.

The effect of pH on the total amount of oxygen bound by solutions of blackfish haemoglobin (Root effect) is shown in Fig. 8. The Root effect is small, as haemoglobin equilibrated with air unloads only about 10% of its total bound oxygen when the pH falls from 7·5 to 6·0. Analysis of swimbladder gas revealed a partial pressure of oxygen of 143 mmHg, a value similar to environmental tensions.

Muscle morphology and biochemistry

Proportions of red to white muscle

The relative proportions of red and white muscle masses in the body musculature of the blackfish, and a number of other fishes of differing swimming behaviour, are shown in Fig. 9. In blackfish, the red muscle is only a minor component accounting for less than 1% of the total propulsive body musculature.

Glycolytic enzymes

The maximum activities of the glycolytic enzymes hexokinase, phosphorylase, phosphofructokinase and lactate dehydrogenase in blackfish white muscle are shown

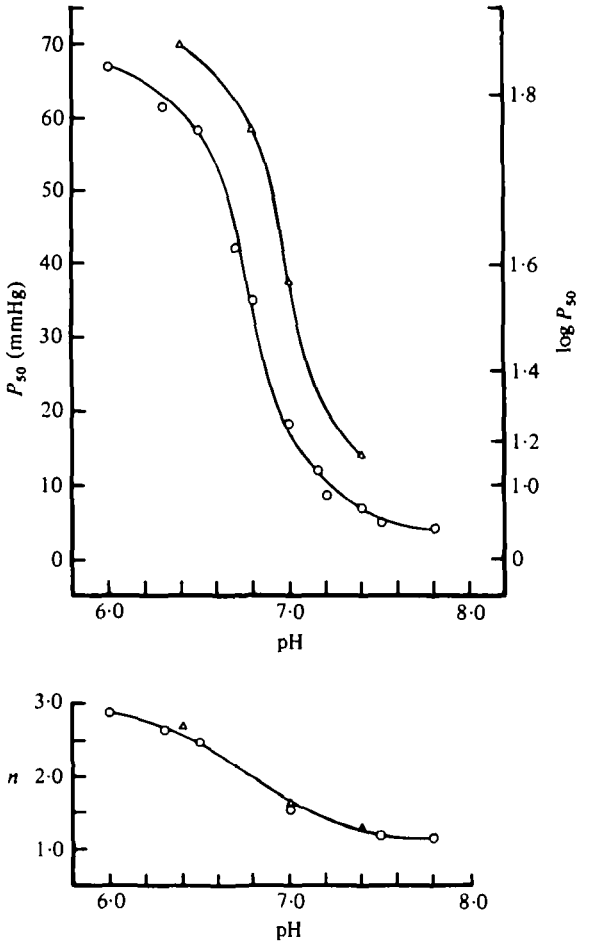

Fig. 7. The effect of pH on the oxygen affinity (P_{50}), and Hill coefficient (n) of solutions of stripped haemoglobin: stripped haemoglobin solution, O; stripped haemoglobin solution with ATP:Hb_4 = 3·0, △. Experimental conditions: assay temperature, 20 °C, equilibration P_{O_2}, 0 mmHg; haemoglobin concentration 1·5 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl. Hill coefficient calculated between 25 % and 75 % HbO_2. Each point was done in triplicate with 0·5 mmHg.

in Table 5. The lactate dehydrogenase activity was not significantly inhibited by high pyruvate concentrations up to 10 mM. Electrophoretic and immunochemical analysis revealed that the M_4 isoenzyme accounted for 92 % of the total lactate dehydrogenase activity present in the tissue.

Production of lactate following exercise

The concentrations of lactate in whole blood and in skeletal muscle of blackfish at rest, and at various times following exercise, are shown in Fig. 11. Muscle lactate increased in a linear fashion throughout the 3 h recovery period, with values rising from 14 to 40 μmole lactate/g wet weight muscle. Whole blood lactate showed quite a different response to that observed for muscle. During the first 30 min of recovery,

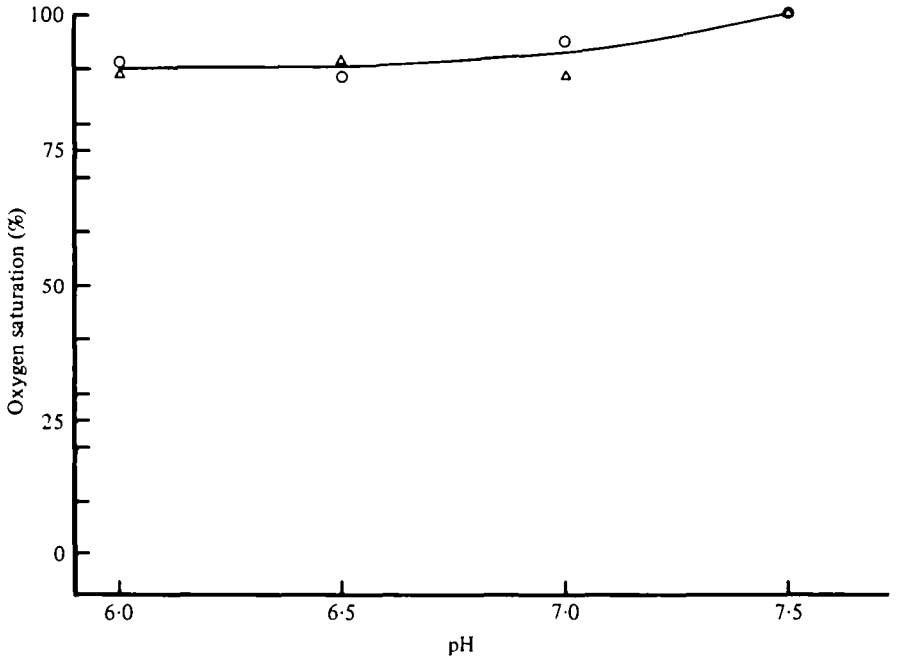

Fig. 8. The effect of pH on the oxygen-carrying capacity of a solution of stripped haemoglobin: stripped haemoglobin solution, ○; stripped haemoglobin solution with ATP:Hb_4 = 3·0, △. Experimental conditions: assay temperature, 20 °C; equilibration P_{O_2}, 0 mmHg; haemoglobin concentration 1·7 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl.

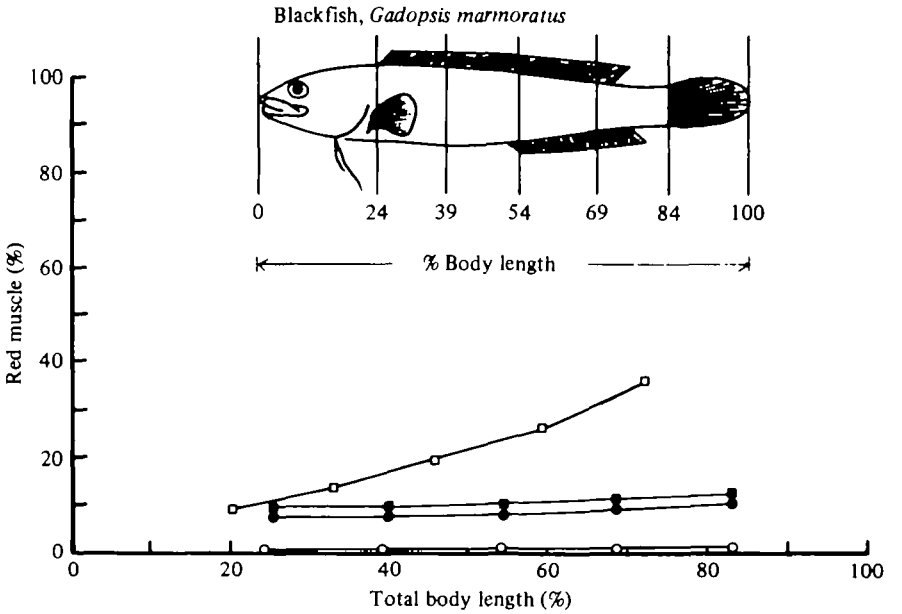

Fig. 9. The proportions of red muscle expressed as a percentage of total propulsive muscle in blackfish and other species of fish: *Gadopsis marmoratus* (blackfish), ○; *Salmo gairdneri* (rainbow trout), ■; *Salvelinus fontinalis* (brook trout), ●; *Sardinops neopilchardus* (sardine), □.

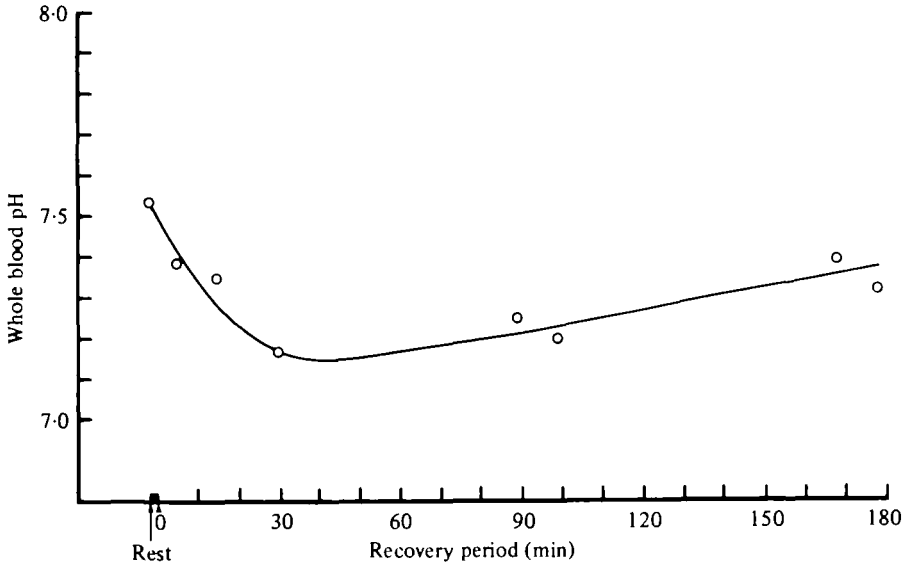

Fig. 10. Whole blood pH of blackfish at rest, and at various times following exercise to exhaustion. Experimental conditions: water temperature 20 °C. Fish were exercised to exhaustion for 2 min. Whole blood pH was measured at 20 °C immediately following blood withdrawal. Each point represents the pH of blood for a single fish, or for blood pooled from 2 fish.

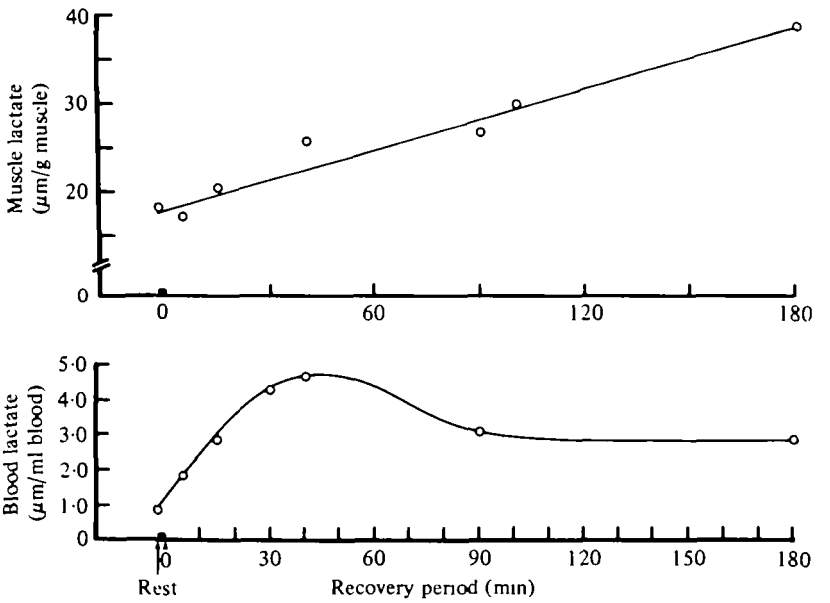

Fig. 11. Lactate concentrations in white muscle and whole blood of blackfish at rest, and at various times following exercise to exhaustion. Each point represents a value for a single fish, or a value for blood pooled from 2 fish. Exercise conditions as for Fig. 10.

Table 5. *Maximum activities of glycolytic enzymes in blackfish white muscle*

Enzyme	Specific activity*
Phosphorylase	$11{\cdot}5 \pm 0{\cdot}6$
Hexokinase	$< 0{\cdot}1$ (3)
Phosphorylase:hexokinase ratio	~ 115
Phosphofructokinase	$13{\cdot}4 \pm 2{\cdot}7$ (3)
Lactate dehydrogenase	407 ± 32 (3)

* Activities expressed as μmole substrate/min g wet weight white muscle at 25 °C.
Values given are the mean ± standard deviation with the number of fish assayed in parentheses.

blood lactate increased from 0·7 to a maximum of 4·6 μmole lactate/ml whole blood, then decreased to a value of about 3·0 μmole/ml and remained at this level during the remainder of the 3 h recovery period, despite the steady rise in muscle lactate.

DISCUSSION

Intraerythrocytic modulation of haemoglobin oxygen affinity

The oxygen equilibrium curves in Fig. 1 clearly demonstrate the importance of intraerythrocytic factors in reducing the high intrinsic oxygen affinity of blackfish haemoglobin into the physiologically relevant range observed for whole blood. The major factors known to regulate haemoglobin function in vertebrates are organic phosphates, pH and CO_2, and the relative importance of these factors in modulating haemoglobin function in blackfish whole blood, are considered in the following discussion.

Organic phosphates

A number of studies have demonstrated the importance of organic phosphates as regulators of haemoglobin oxygen affinity in fish erythrocytes. Evidence for their role is threefold: the presence within the erythrocyte of high concentrations of organic phosphate compounds, often exceeding the molar concentration of haemoglobin (Bartlett, 1976, 1978, 1980); the ability of physiological concentrations of these naturally occurring phosphates to lower haemoglobin oxygen affinity *in vitro*, in a manner similar to that displayed by 2,3-DPG with human blood (Gillen & Riggs, 1971; Weber & De Wilde, 1975); and finally, correlations between changes in the levels of these organic phosphates and changes in whole blood oxygen affinity in fish subjected to hypoxia (Wood & Johansen, 1972; Weber, Lykkeboe & Johansen, 1975, 1976; Weber & Lykkeboe, 1978; Greaney & Powers, 1978; Wood, 1980), and thermal acclimation (Powers, 1974; Wood, 1980; Dobson & Baldwin, 1982).

The presence of high concentrations of ATP and lower concentrations of GTP in blackfish erythrocytes (Table 3, Fig. 2), together with their pronounced effect on the oxygen affinity of haemoglobin solutions at physiological molar ratios and intraerythrocytic pH (Fig. 4), indicates that these organic phosphates are the major regulators of oxygen affinity in blackfish blood.

Carbon dioxide

The ability of increased P_{CO_2} to lower blood oxygen affinity may be elicited through two separate mechanisms; firstly, the effect of CO_2 to reduce intraerythrocytic pH via the carbonic acid system, giving rise to the Bohr effect, and secondly, the direct combination of CO_2 with haemoglobin as bound carbamate, which lowers the affinity of haemoglobin for oxygen (Henriques, 1928; Kilmartin & Rossi-Bernardi, 1973).

While increasing P_{CO_2} markedly lowers the oxygen affinity of blackfish whole blood, it does so only through the effect of pH, as no such change in oxygen affinity was observed with strongly pH-buffered haemoglobin solutions. To our knowledge, this lack of oxygen-linked CO_2 binding has not been reported for any other naturally occurring haemoglobins, that is haemoglobins that have not been chemically modified. In mammals, CO_2 binds preferentially with the terminal α-amino groups of the β-chain of deoxyhaemoglobin at physiological pH (Rossi-Bernardi & Roughton, 1967; Kilmartin & Rossi-Bernardi, 1969, 1973; Arnone, 1972; Perella, Bresciani & Rossi-Bernardi, 1975; Bauer & Kurtz, 1977). Since the α-terminal amino groups of the β-chains also bind organic phosphates (Bauer, 1969, 1970; Perutz, 1970; Arnone, 1972), competition for a common binding site explains the antagonistic effect of CO_2 on the binding of 2,3-DPG (Bunn & Briehl, 1970). Similar competition between CO_2 and organic phosphates has been reported for carp haemoglobin (Weber & Lykkeboe, 1978). However, competition studies with blackfish haemoglobin clearly demonstrate that ATP binding is insensitive to CO_2 (Table 4). The lack of oxygen-linked CO_2 binding site(s) on blackfish haemoglobin indicates not only that the terminal α-amino groups of the α-chains are blocked, as occurs with a number of other teleost fish haemoglobins (Riggs, 1970; Weber & Lykkeboe, 1978), but also that the terminal α-amino groups of the β-chain must be blocked or modified to prevent CO_2 binding.

The physiological significance of direct oxygen-linked CO_2 binding to most vertebrate haemoglobins is not well understood (Kilmartin, 1976; Farmer, 1979). As water-breathing fish have very low circulating CO_2 tensions compared with air-breathers (Rahn, 1966), carbamate formation can make little contribution to oxygen or CO_2 transport. The absence of oxygen-linked CO_2 binding with blackfish haemoglobin at physiological pH supports this view.

Hydrogen ion concentration

Blackfish whole blood and haemoglobin solutions are very sensitive to changes in pH, with a decreasing pH leading to a reduction in oxygen affinity. The functional significance of the alkaline Bohr effect is thought to lie in enhancing oxygen delivery to the tissues at times of greatest oxygen demand. During muscle work, acidic metabolites will wash out into the blood and lower pH, and the subsequent uptake of hydrogen ions by haemoglobin causes the release of more bound oxygen than would occur merely in response to the lowering of tissue PO_2 during activity.

Relationships between habitat and swimming behaviour, and oxygen-binding properties of whole blood

The freshwater blackfish is largely a bottom-dwelling animal frequenting snags and fallen timber in the slower sections of well-oxygenated streams in south-eastern Australia. General body shape, and muscle morphology and biochemistry are consistent with the observed swimming behaviour in nature and laboratory aquaria. The elongated rounded body tapering gradually to the caudal peduncle (Fig. 9) is characteristic of short-burst performers (Webb, 1978) and this, together with the high percentage of white muscle (99%), suggests predator–prey interactions involving short-term bursts of high-speed swimming, rather than prolonged periods of steady-state cruising. In the laboratory, fish can be easily exhausted in about 1 min of continuous stimulation, and this lack of endurance is reflected in their poor fighting abilities when captured by rod and line.

The high activities of phosphorylase relative to hexokinase indicate that the white muscle utilizes anaerobic glycolysis with glycogen as the major fuel. The predominance of the M_4 lactate dehydrogenase isoenzyme, which is not significantly inhibited at high pyruvate concentrations, also suggests that the muscle is set up to channel glycolytically derived pyruvate to lactate during bursts of anaerobic work. The maximum activities of phosphorylase and phosphofructokinase are similar and provide for a maximum glycolytic flow of about 26 μmole lactate/min.g wet weight muscle. This value is fourfold lower than those calculated from phosphofructokinase activities in white muscle of rainbow trout and dogfish (Crabtree & Newsholme, 1975), and suggests that although blackfish rely upon anaerobic glycolysis during rapid swimming, their anaerobic capabilities are rather limited.

In general, the oxygen-binding properties of fish blood reflect oxygen availability, with high oxygen affinity found in species that encounter hypoxic environments, and lower oxygen affinities in species inhabiting well-oxygenated waters (Powers *et al.* 1979; Riggs, 1979; Powers, 1980). The low-affinity blood of blackfish compared with other fish bloods (Table 1), suggests possible disadvantages during oxygen loading at the gills. However, this condition is presumably circumvented by the preferred habitat of the animal, a highly oxygenated environment with oxygen tensions between 130 and 140 mmHg (G. P. Dobson, unpublished data). Indeed, this low-affinity blood probably places severe limitations on the ability of blackfish to frequent and survive in waters low in oxygen without major physiological adjustments.

A second important feature of the whole blood oxygen equilibrium curve is the shape, which determines the functional range of oxygen tensions over which blood can unload oxygen to the tissues. Active fish utilizing sustained aerobic muscle work, like the salmonid species, generally display a distinctly sigmoidal curve with n values approaching 2·5–3·0, while less active species like the cyprinids are characterized by a more hyperbolic-shaped curve with lower n values. The hyperbolic curve with an n value of 1·58 obtained for blackfish at 20 °C further justifies placing this animal among the less active species of fish.

Muscle activity associated with swimming can have a profound effect on the oxygen-binding properties of blood by increasing the concentration of hydrogen ions inside

the red cell as a result of the hydration of CO_2 and the influx of hydrogen ions from the plasma following washout from those tissues undergoing anaerobiosis. Exercising blackfish to complete exhaustion has the immediate effect of lowering blood pH (Fig. 10), with the maximum decrease occurring after 30 min recovery. This decrease can be correlated with increased muscle anaerobiosis (Fig. 11), but it should be stressed that the initial decrease in blood pH following activity is probably due to the rapid rise in P_{CO_2} (Piiper, Meyer & Drees, 1972; Wood, McMahon & McDonald, 1977).

An interesting feature of the exercise experiment is that changes in blood lactate do not reflect muscle lactate concentrations. The accumulation and retention of high concentrations of muscle lactate, relative to blood, following exercise (Fig. 11), appears to be a general property of fish white muscle (Black *et al.* 1962; Driedzic & Hochachka, 1978; Wardle, 1978). This phenomenon is thought to reflect the unfavourable transfer conditions resulting from the low capillary density and low perfusion rates of white muscle. The continued increase in muscle lactate concentrations for at least 3 h after exercise suggests that blackfish continues to utilize anaerobic glycolysis, rather than aerobic metabolism, to meet the energy demands of the muscle during the recovery phase. The possibility exists that the continuation of anaerobic glycolysis after exercise may be important to maintain cellular transport processes and assist in the regeneration of creatine phosphate stores in white muscle. The time it takes to switch from predominantly anaerobic to predominantly aerobic metabolism in blackfish in recovery is not known. Similarly, the retention of high lactate concentrations and the eventual fate of lactate in fish white muscle following activity is not well understood.

We conclude that the oxygen-binding properties of blackfish whole blood are not adapted to supply large amounts of oxygen to sustain high levels of aerobic metabolism but, rather, that they are adjusted to maintain adequate tissue oxygenation for slow manoeuvring aerobic swimming in waters of high oxygen tension. The large Bohr effect may be an important adaptation to maximize oxygen delivery to oxygen-dependent organs and tissues following short-term bursts of high-speed swimming associated with the capture of prey and avoiding predators.

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REFERENCES

AMES, B. N. & DUBIN, D. T. (1960). The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. biol. Chem.* **235**, 769–775.

ARNONE, A. (1972). X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin. *Nature, Lond.* **237**, 146–149.

ASTRUP, P., ENGEL, K., SEVERINGHAUS, J. W. & MUNSON, E. (1965). The influence of temperature and pH on the dissociation curve of oxyhaemoglobin of human blood. *Scan. J. clin. Lab. Invest.* **17**, 515–523.

BALDWIN, J. & SEYMOUR, R. S. (1977). Adaptation to anoxia in anakes: levels of glycolytic enzymes in skeletal muscle. *Aust. J. Zool.* **25**, 9–13.

BARTLETT, G. R. (1976). Phosphate compounds in red cells of reptiles, amphibians and fish. *Comp. Biochem. Physiol.* **55** A, 211–214.

BARTLETT, G. R. (1978). Water-soluble phosphates of fish red cells. *Can. J. Zool.* **56**, 870–877.

BARTLETT, G. R. (1980). Phosphate compounds in vertebrate red cells, *Am. Zool.* **20**, 103–114.

BAUER, C. (1969). Antagonistic influence of CO_2 and 2,3-diphosphoglycerate on the Bohr effect of human haemoglobin. *Life Sci.* **8**, 1041–1046.

BAUER, C. (1970). Reduction of the carbon dioxide affinity of human haemoglobin solutions by 2,3-diphosphoglycerate. *Respir. Physiol.* **10**, 10–19.

BAUER, C. & KURTZ, A. (1977). Oxygen-linked CO_2 binding to isolated β-subunits of human haemoglobin. *J. biol. Chem.* **252**, 2952–2955.

BENESCH, R. & BENESCH, R. E. (1967). The effect of organic phosphates from the human erythrocyte on the allosteric properties of haemoglobin. *Biochem. biophys. Res. Commun.* **26**, 162–167.

BERGMEYER, H. U. (1974) (ed.). *Methods of Enzymatic Analysis*, 2nd ed., pp. 1464–1468. New York: Academic Press.

BLACK, E. C. (1940). The transport of oxygen by the blood of freshwater fish. *Biol. Bull. mar. biol. Lab., Woods Hole* **79**, 215–219.

BLACK, E. C., CONNOR, A. R., LAM, K. C. & CHIU, W. (1962). Changes in glycogen, pyruvate and lactate in rainbow trout (*Salmo gairdneri*) during and following severe muscular activity. *J. Fish. Res. Bd Can.* **19**, 409–436.

BLACK, E. C., KIRKPATRIC, D. & TUCKER, H. H. (1966). Oxygen dissociation curves of the blood of brook trout (*Salvelinus fontinalis*) acclimated to summer and winter temperatures. *J. Fish. Res. Bd Can.* **23**, 1–13.

BONAVENTURA, C., SULLIVAN, B., BONAVENTURA, J. & BRUNORI, M. (1976). Spot haemoglobin. Studies on the Root effect haemoglobin of a marine teleost. *J. biol. Chem.* **251**, 1871–1876.

BUNN, H. F. & BRIEHL, R. W. (1970). The interaction of 2,3-disphosphoglycerate and various human haemoglobins. *J. clin. Invest.* **49**, 1088–1095.

CAMERON, J. N. (1971). Oxygen dissociation characteristics of the blood of the rainbow trout *Salmo gairdneri*). *Comp. Biochem. Physiol.* **38** A, 699–704.

CHANUTIN, A. & CURNISH, R. R. (1967). Effect of organic and inorganic phosphates on the oxygen equilibrium curve of human erythrocytes. *Archs Biochem. Biophys.* **121**, 96–102.

CRABTREE, B. & NEWSHOLME, E. A. (1975). Comparative aspects of fuel utilization and metabolism by muscle. In *Insect Muscle* (ed. P. N. R. Usherwood), pp. 405–500. New York: Academic Press.

DIJKHUIZEN, P., BUURSMA, A., FONGERS, T. M. E., GERDING, A. M., OESEBURG, B. & ZIJLSTRA, W. G. (1977). The oxygen binding capacity of human haemoglobin: Hüfner's factor redetermined. *Pflügers Arch. ges. Physiol.* **369**, 223–231.

DILL, D. B. & FORBES, W. H. (1941). Respiratory and metabolic effects of hypothermia. *Am. J. Physiol.* **132**, 685–697.

DOBSON, G. P. & BALDWIN, J. (1982). Regulation of blood oxygen affinity in the Australian blackfish *Gadopsis marmoratus* II. Thermal acclimation. *J. exp. Biol.* **99**, 245–254.

DRIEDZIC, W. R. & HOCHACHKA, P. W. (1978). Metabolism in fish during exercise. In *Fish Physiology*, vol. 7 (eds. W. S. Hoar and D. R. Randall), pp. 503–543. New York: Academic Press.

EDDY, F. B. (1973). Oxygen dissociation curves of the blood of the tench, *Tinca tinca*. *J. exp. Biol.* **58**, 281–293.

EDWARDS, M. J. & MARTIN, R. J. (1966). Mixing technique for the oxygen–haemoglobin equilibrium and Bohr effect. *J. appl. Physiol.* **21**, 1898–1902.

ENOKI, Y., TOMITA, S., MAEDA, N., KAWASE, M. & OKUDA, T. (1972). A simple method for determination of red cell intracellular pH. *J. Physiol. Soc. Japan* **34**, 761–762.

EVELYN, K. A. & MALLOY, H. T. (1938). Microdetermination of oxyhaemoglobin, methaemoglobin and sulfhaemoglobin in a single sample of blood. *J. biol. Chem.* **126**, 665–662.

FARMER, M. (1979). The transition from water to air breathing: effects of CO_2 on haemoglobin function. *Comp. Biochem. Physiol.* **62** A, 109–114.

GILLEN, R. G. & RIGGS, A. (1971). The haemoglobins of a freshwater teleost, *Cichlasoma cyanoguttatum* (Baird and Girard). I. The effects of phosphorylated organic compounds upon the oxygen equilibria. *Comp. Biochem. Physiol.* **38** B, 585–595.

GREANEY, G. S. & POWERS, D. A. (1978). Allosteric modifiers of fish haemoglobins: *in vitro* and *in vivo* studies of the effect of ambient oxygen on erythrocyte ATP concentrations. *J. exp. Zool.* **203**, 339–350.

GRIGG, G. C. (1969). Temperature induced changes in the oxygen equilibrium curve of the blood of the brown bullhead, *Ictalarus nebulosus*. *Comp. Biochem. Physiol.* **28**, 1203–1223.

GRIGG, G. C. (1974). Respiratory function of blood in fishes. In *Chemical Zoology*, vol. 8 (ed. M. Florkin and B. J. Scheer), pp. 331–368. New York: Academic Press.

HAAB, P. E., PIIPER, J. & RAHN, H. (1960). Simple method for rapid determination of an O_2-dissociation curve of blood. *J. appl. Physiol.* **15**, 1148–1149.

HAWS, T. G. & GOODNIGHT, C. J. (1962). Some aspects of the haematology of two species of catfish in relation to their habits. *Physiol. Zool.* **35**, 8–17.

HENRIQUES, C. M. (1928). Die Bindungsweise des kohlendioxyds im Blute. I–V. *Biochem. Z.* **200**, 1–24.

HOLMES, R. S. & SCOPES, R. K. (1974). Immunochemical homologies among Vertebrate lactate-dehydrogenase Isozymes. *Eur. J. Biochem.* **43**, 167–177.

IRVING, L., BLACK, E. C. & SAFFORD, V. (1941). The influence of temperature upon the combination of oxygen with the blood of trout. *Biol Bull. Mar. biol. Lab., Woods Hole* **80**, 1–17.

ISAAKS, R. E., HARKNESS, D. R., FROEMAN, G. A. & SUSSMAN, S. A. (1976). Studies on avian erythrocyte metabolism. I. Procedure for separation and quantitation of the major phosphorylated metabolic intermediates by anion-exchange chromatography. *Comp. Biochem. Physiol.* **53**A, 95–99.

JELKMAN, W. & BAUER, C. (1976). What is the best method to remove 2,3-diphosphoglycerate from haemoglobin? *Analyt. Biochem.* **75**, 382–388.

JOHANSEN, K. & LENFANT, C. (1972). A comparative approach to the adaptability of O_2-Hb affinity. In *Oxygen Affinity of Haemoglobin and Red Cell Acid-Base status:* Alfred Benzon Symposium, vol. 4 (ed. M. Rorth and P. Astrup), pp. 750–780. Copenhagen: Munksgaard.

KILMARTIN, J. V. (1976). Interactions of haemoglobin with protons, CO_2 and 2,3-diphosphoglycerate. *Brit. Med. Bull.* **32**, 209–212.

KILMARTIN, J. V. & ROSSI-BERNARDI, L. (1969). Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups. *Nature, Lond.* **222**, 1243–1246.

KILMARTIN, J. V. & ROSSI-BERNARDI, L. (1973). Interactions of haemoglobin with hydrogen ions, carbon dioxide and organic phosphates. *Physiol. Rev.* **53**, 836–890.

KROGH, A. & LEITCH, I. (1919). The respiratory function of the blood in fishes. *J. Physiol.* **52**, 288–300.

MOSSE, P. R. L. & HUDSON, R. C. L. (1977). The functional roles of different muscle fibre types identified in the myotomes of marine teleosts: a behavioural, anatomical and histological study. *J. Fish. Biol.* **11**, 417–430.

MULLER, B. D. & BALDWIN, J. (1978). Biochemical correlates of flying behaviour in bats. *Aust. J. Zool.* **26**, 29–37.

MURPHY, W. S., METCALF, J., HOVERSLAND, A. S. & DHINDSA, D. S. (1977). Postnatal changes in blood respiratory characteristics in an American opossum (*Didelphis virginiana*). *Respir. Physiol.* **29**, 73–80.

PERELLA, M., BRESCIANI, D. & ROSSI-BERNARDI, L. (1975). The binding of CO_2 to human haemoglobin. *J. biol. Chem.* **250**, 5413–5418.

PERUTZ, M. F. (1970). The Bohr effect and combination with organic phosphates. *Nature, Lond.* **228**, 734–739.

PIIPER, J., MEYER, M. & DREES, F. (1972). Hydrogen balance in the elasmobranch, *Scyliorhinus stellaris* after exhaustive activity. *Respir. Physiol.* **16**, 290–303.

POWERS, D. A. (1974). Structure, function and molecular ecology of fish haemoglobins. *Ann. N.Y. Acad. Sci.* **241**, 472–490.

POWERS, D. A. (1980). Molecular ecology of teleost fish haemoglobins: strategies for adapting to changing environments. *Am. Zool.* **20**, 139–162.

POWERS, D. A., FYHN, H. J., FYHN, U. E. H., MARTIN, J. P., GARLICK, R. L. & WOOD, S. C. (1979). A comparative study of the oxygen equilibria of blood from 40 genera of Amazonian fishes. *Comp. Biochem. Physiol.* **62**A, 67–85.

RAHN, H. (1966). Aquatic gas exchange: theory. *Respir. Physiol.* **1**, 1–2.

REDFIELD, A. C. (1933). The evolution of the respiratory function of the blood. *Quart. Rev. Biol.* **8**, 31–57.

RIGGS, A. (1970). Properties of fish haemoglobins. In *Fish Physiology*, vol. 4 (ed. W. S. Hoar and D. R. Randall), pp. 209–252. New York: Academic Press.

RIGGS, A. (1979). Studies of the haemoglobins of Amazonian fishes: an overview. *Comp. Biochem. Physiol.* **62**A, 257–272.

ROSSI-BERNARDI, L. & ROUGHTON, F. J. W. (1967). The specific influence of carbon dioxide and carbamino compounds on the buffer power and Bohr effect of human haemoglobin. *J. Physiol.* **189**, 1–29.

VAN KAMPEN, E. J. & ZIJLSTRA, W. G. (1965). Determination of haemoglobin and its derivatives. *Adv. clin. Chem.* **8**, 141–187.

WARDLE, C. S. (1978). Non-release of lactic acid from anaerobic swimming muscle of plaice, *Pleuronectes platessa* L. A stress reaction. *J. exp. Biol.* **77**, 141–155.

WEBB, P. W. (1978). Fast-start performance and body form in seven species of teleost fish. *J. exp. Biol.* **74**, 211–226.

WEBER, R. E. & DE WILDE, J. A. M. (1975). Oxygenation properties of haemoglobins from the flatfish plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*). *J. comp. Physiol.* **101**, 99–110.

WEBER, R. E. & LYKKEBOE, G. (1978). Respiratory adaptations in carp blood: influence of hypoxia, red cell organic phosphate, divalent cations and CO_2 on haemoglobin–oxygen affinity. *J. comp. Physiol.* **128**, 127–137.

WEBER, R. E., LYKKEBOE, G. & JOHANSEN, K. (1975). Biochemical aspects of the adaptation of haemoglobin–oxygen affinity of eels to hypoxia. *Life Sci.* **17**, 1345–1349.

WEBER, R. E., LYKKEBOE, G. & JOHANSEN, K. (1976). Physiological properties of eel haemoglobin: hypoxic acclimation, phosphate effects and multiplicity. *J. exp. Biol.* **64**, 75–88.

WILLMER, E. N. (1934). Some observations on the respiration of certain tropical freshwater fishes. *J. exp. Biol.* **11**, 283–306.

WOOD, S. C. (1980). Adaptation of red blood cell function to hypoxia and temperature in ectothermic vertebrates. *Am. Zool.* **20**, 163–172.

WOOD, S. C. & JOHANSEN, K. (1972). Adaptation to hypoxia by increased Hb–O_2 affinity and decreased red cell ATP concentration. *Nature, New Biol.* **237**, 278–279.

WOOD, S. C. & LENFANT, C. (1979). Oxygen transport and oxygen delivery. In *Evolution of Respiratory Processes: a Comparative Approach.* (ed. S. C. Wood and C. Lenfant), pp. 193–224. New York: Marcel Dekker.

WOOD, C. S., MCMAHON, B. R. & MCDONALD, D. G. (1977). An analysis of changes in blood pH following exhaustive activity in the starry flounder, *Platichthys stellatus*. *J. exp. Biol.* **69**, 173–185.