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

II. THERMAL ACCLIMATION

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SUMMARY

1. The effects of thermal acclimation on whole blood oxygen affinity were examined in the freshwater blackfish *Gadopsis marmoratus*.

2. Oxygen equilibrium curves for 20 °C-acclimated fish were shifted to the right of curves obtained for 10 °C-acclimated fish when determined at both 20 °C and 10 °C. Oxygen equilibrium curves obtained for solutions of stripped haemoglobin prepared from blood of 20 °C- and 10 °C-acclimated fish did not show the differences observed for whole blood.

3. Thermal acclimation did not alter the number, migration rates, or relative amounts of the five electrophoretic forms of haemoglobin present in blackfish blood.

4. The intraerythrocytic concentration of nucleoside triphosphates was higher in the 20 °C-acclimated fish than in 10 °C-acclimated fish, while the whole blood haemoglobin concentration was lower in the 20 °C-acclimated fish. These differences gave NTP:Hb_4 molar ratios of 1·68 for the 20 °C-acclimated fish and 1·32 for 10 °C-acclimated fish. The effects of nucleoside triphosphates on oxygen affinity were similar for stripped haemoglobins of both acclimation groups.

5. The change in NTP:Hb_4 molar ratios with acclimation temperature acts to enhance oxygen unloading to the tissues rather than oxygen uptake at the gills at the higher acclimation temperature. As the waters inhabited by the blackfish retain high oxygen tensions at 20 °C, these changes in blood oxygen affinity could be considered adaptive if they were associated with elevated rates of oxygen-dependent metabolism at the higher temperatures.

INTRODUCTION

Many aspects of respiratory function in water-breathing fish are influenced by environmental temperature. Changes in water temperature not only alter environmental oxygen availability and oxygen-dependent metabolic rate functions (Fry, 1957, 1971), but also have profound effects upon the oxygen transport properties of blood. These latter effects may arise directly through temperature-induced changes in the

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oxygen affinity of the haemoglobin molecule, or more indirectly via changes in the various specific intraerythrocytic modulators of haemoglobin function (Grigg, 1969; Powers, 1974; Greaney & Powers, 1978; Wood & Lenfant, 1979).

Fish utilize a number of different adaptive mechanisms for adjusting the oxygen delivery system to meet tissue oxygen demands throughout the range of habitat temperatures normally encountered. Short-term responses to temperature change usually involve alterations in rate of gill ventilation and blood circulation (Randall & Shelton, 1963; Randall & Smith, 1967; Randall, 1970; Hughes & Roberts, 1970). However, as the energetic costs of maintaining high rates of water breathing at higher temperatures may become prohibitive (see Jones, 1971), a more efficient long-term strategy is to change the oxygen transport properties of blood. This may be achieved by altering parameters such as haemoglobin content (Houston & De Wilde, 1968; Cameron, 1970; Powers, 1974; Houston & Smeda, 1979), the molecular forms of haemoglobin present (Houston & Cry, 1974; Weber, Wood & Lomholt, 1976), and the concentrations of organic phosphates and hydrogen ions which influence the oxygen-binding properties of haemoglobin (Powers, 1974; Wood *et al.* 1978).

The present study was undertaken to investigate the effects of thermal acclimation and seasonal acclimatization on the oxygen transport properties of blood in the freshwater blackfish *Gadopsis marmoratus*. In an accompanying paper (Dobson & Baldwin, 1982) it was reported that the most important regulators of whole blood oxygen affinity in this fish are nucleoside triphosphates and pH. Following thermal acclimation, changes in the whole blood oxygen affinity act to enhance the unloading of oxygen to the tissues at higher temperatures. The changes in blood oxygen affinity with acclimation temperature result from subtle alterations in the nucleoside triphosphate: haemoglobin (NTP:Hb_4) molar ratios.

MATERIALS AND METHODS

Blackfish were collected and maintained as described in the accompanying paper (Dobson & Baldwin, 1982). Winter fish were held at 10 °C, and summer fish at 20 °C, for at least 4 weeks in constant-temperature rooms set on a 12 h light/12 h dark cycle. These acclimation temperatures approximated water temperatures in the river at the time of capture.

All experimental procedures used in this study, with the exception of the electrophoretic separation of haemoglobins and blood bicarbonate determinations, have been described in detail in Dobson & Baldwin (1982). The molecular heterogeneity of blackfish haemoglobins was studied using polyacrylamide gel disc electrophoresis as described by Dietz, Lubrano & Rubinstein (1971), after Davis (1962) and Ornstein (1964). Electrophoresis was carried out on 5·5% gels with tris-glycine tank buffer, pH 8·9. Haemolysates were prepared from saline-washed erythrocytes lysed by vigorous mixing with 2 vols. of ice-cold distilled water, and centrifuged at 4000 *g* and 4 °C for 5 min. The supernatant was converted to the more stable carbonmonoxy form by saturating with carbon monoxide. Prior to loading, 1 drop of 5% sucrose was added to a haemolysate sample containing 400 μg haemoglobin in 10 μl. Following electrophoresis the gels were scanned without staining on a recording densitometer (Chromoscan, Joyce, Loeb and Co. England).

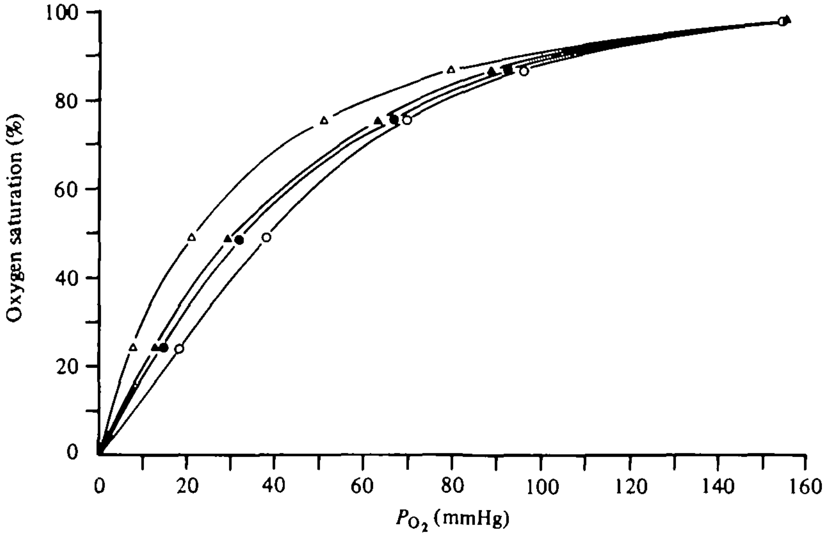

Fig. 1. Comparison of whole blood oxygen equilibrium curves for 10 °C- and 20 °C-acclimated blackfish, determined at 10 °C and 20 °C; 10 °C-acclimated fish at 10 °C, △; 10 °C-acclimated fish at 20 °C, ▲; 20 °C-acclimated fish at 10 °C, ●; 20 °C-acclimated fish at 20 °C, ○. Experimental conditions: 20 °C-acclimated fish. Blood pooled from nine fish; whole blood haemoglobin concentration 5·24 g/100 ml; whole blood pH 7·52 at 20 °C. 10 °C-acclimated fish. Blood pooled from 15 fish; whole blood haemoglobin concentration 5·92 g/100 ml; whole blood pH 7·70 at 10 °C. The experiment was repeated and each point had ±1 mmHg variability. Equilibration P_{CO_2}, 3·95 mmHg.

Blood bicarbonate concentrations were determined from the *in vivo* mixed arterial-venous blood pH and P_{CO_2} measurements. These values were substituted into the Henderson–Hasselbalch equation and bicarbonate concentration calculated using the pK' and α_{CO_2} values for human plasma (Severinghaus, 1956). The use of these human values for fish blood assumes however that the plasma ionic strengths for both are similar (Albers, 1970).

RESULTS

Effects of thermal acclimation on the oxygen equilibrium curves of whole blood and haemoglobin solutions

The oxygen equilibrium curves obtained for whole blood of 10 °C- and 20 °C-acclimated blackfish, determined at both 10 °C and 20 °C, are plotted in Fig. 1. Comparisons between these four curves show that the oxygen equilibrium curves for the 20 °C-acclimated fish are shifted to the right of those obtained for the 10 °C-acclimated fish, at both assay temperatures. At 20 °C the P_{50} value for the 20 °C-acclimated fish is 8·5 mmHg higher than for the 10 °C-acclimated fish. At 10 °C this difference is 10·5 mmHg.

Whole blood samples collected from fish immediately after capture in winter and in summer gave P_{50} values that were the same as those obtained for animals acclimated to 10 °C and 20 °C respectively in the laboratory.

Accompanying the shift in oxygen affinity with acclimation or acclimatization

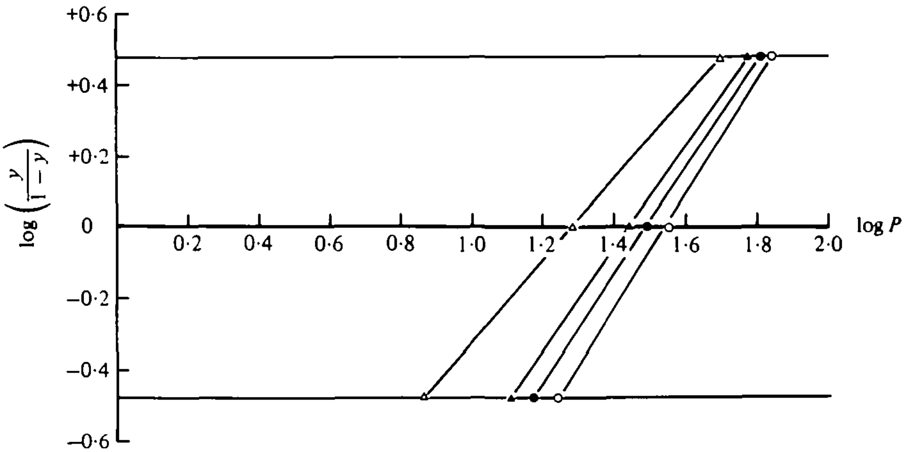

Fig. 2. The Hill coefficient, *n*, calculated between 25 % and 75 % HbO_2 for the whole blood equilibrium curves plotted in Fig. 1: 10 °C-acclimated fish at 10 °C, △; 10 °C-acclimated fish at 20 °C, ▲; 20 °C-acclimated fish at 10 °C, ●; 20 °C-acclimated fish at 20 °C, ○. *y* = oxygen saturation of whole blood; *p* = partial pressure of oxygen.

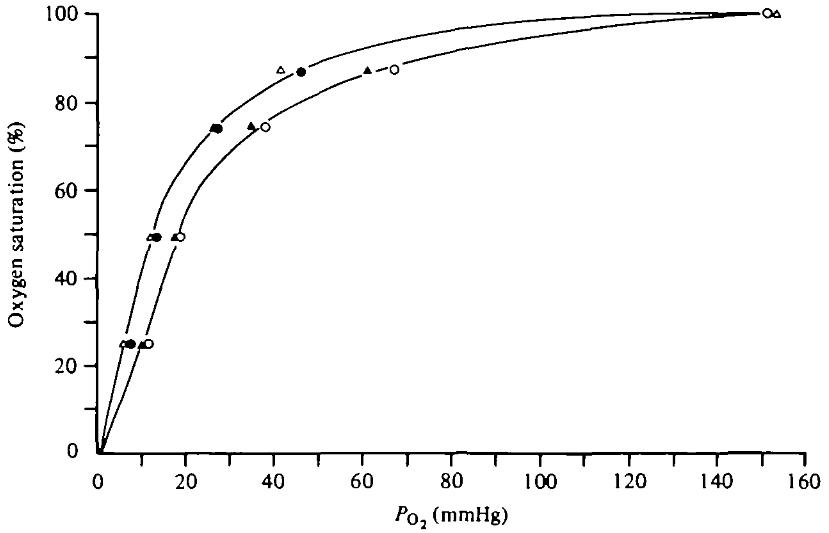

Fig. 3. Comparison of the oxygen equilibrium curves for solutions of stripped haemoglobin from 10 °C- and 20 °C-acclimated blackfish determined at 10 °C and 20 °C: 10 °C-acclimated fish at 10 °C, △; 10 °C-acclimated fish at 10 °C, ▲; 20 °C-acclimated fish at 10 °C, ●; 20 °C-acclimated fish at 20 °C, ○. Experimental conditions: equilibration P_{CO_2}, 3·95 mmHg; haemoglobin concentration, 1·5 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl, pH 7·0 at both 10 °C and 20 °C. *Each point determination had ± 1 mmHg variability.*

temperature is a change in the Hill coefficient, *n* (Fig. 2). The blood of 10 °C-acclimated fish has *n* values of 1·18 at 10 °C and 1·40 at 20 °C, compared to the higher values of 1·49 and 1·58 at 10 °C and 20 °C respectively for the 20 °C-acclimated fish.

To determine whether the marked difference in oxygen affinity of whole blood with thermal acclimation was due to changes in the intrinsic binding properties of haemoglobin, or to changes in the environment in which haemoglobin functions, the dete

Table 1. *Effect of thermal acclimation on respiratory and acid–base properties of whole blood*

Parameter	10 °C-acclimated blackfish at 10 °C	20 °C-acclimated blackfish at 20 °C
Whole blood haemoglobin concentration (g/100 ml)	5·74 ± 0·67 (12)	5·19 ± 0·33 (*S**) (13)
Methaemoglobin concentration (% of total haemoglobin)	5·41 ± 2·73 (12)	4·20 ± 2·45 (15)
Total NTP† (μmole/ml rbc)	5·09 ± 0·68 (12)	6·43 ± 0·53 (*S*) (13)
NTP:Hb_4	1·32 (12)	1·68 (*S*) (13)
ATP:GTP ratio	5:1	5:1
Whole blood pH	7·72 ± 0·09 at 10 °C (18) 7·53 ± 0·03 at 20 °C	7·50 ± 0·08 at 20 °C (21) 7·70 ± 0·02 at 10 °C
Intraerythrocytic pH	7·21 ± 0·02 (8)	7·05 ± 0·03 (*S*) (10)
Mixed arterial-venous whole blood P_{CO_2} (mmHg) (resting fish)	4·0 ± 0·50 (6)	3·5 ± 0·50 (N.S.) (5)
Blood bicarbonate (mM)	7·44 ± 0·65 (6)	3·66 ± 0·54 (*S*) (5)

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

* Significance of the difference between two means as tested by Student's *t* test with a probability level of 0·05. *S* = significant. N.S. = not significant.

† Nucleoside triphosphate.

mination of the four oxygen equilibrium curves shown in Fig. 1 was repeated, using stripped haemoglobin solutions prepared from blood of the two thermal acclimation groups (Fig. 3). In this experiment, essentially identical curves were obtained for both 10 °C- and 20 °C-acclimated fish.

Effects of thermal acclimation on the electrophoretic forms of haemoglobin

Electrophoretic analysis of haemoglobins in the blood of 10 °C- and 20 °C-acclimated blackfish revealed the presence of three major and two minor components. The number, migration rates and relative intensities of these bands were not altered by thermal acclimation.

Effects of thermal acclimation on interactions between haemoglobin and intraerythrocytic nucleoside triphosphates

The concentration of total nucleoside triphosphates differed significantly between the two thermal acclimation groups, with higher values for 20 °C-acclimated fish (Table 1). Anion-exchange chromatography of erythrocyte extracts showed that the total nucleoside triphosphate concentration was made up of ATP and GTP in a ratio of 5:1 in both acclimation groups. Whole blood haemoglobin concentration was less at the higher acclimation temperature, yielding total nucleoside triphosphate concentration:haemoglobin tetramer (NTP:Hb_4) molar ratios of 1·32 and 1·68 respectively for the 10 °C- and 20 °C-acclimated fish (Table 1).

The effects of ATP on the oxygen affinity of solutions of stripped haemoglobin from the two acclimation groups are shown in Fig. 4.

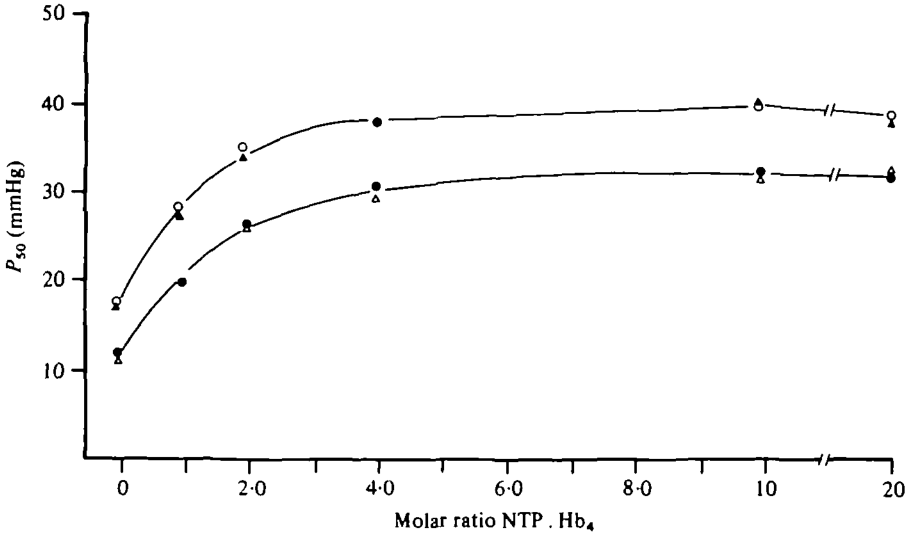

Fig. 4. Effect of ATP on the oxygen affinity (P_{50}) of solutions of stripped haemoglobin from 10 °C- and 20 °C-acclimated blackfish, determined at 10 °C and 20 °C: 10 °C-acclimated fish at 10 °C, △; 10 °C-acclimated fish at 20 °C, ▲; 20 °C-acclimated fish at 10 °C, ●; 20 °C-acclimated fish at 20 °C, ○. Experimental conditions: equilibration P_{O_2}, 0 mmHg; haemoglobin concentration, 1·0 g/100 ml in 0·05 M-bis-tris HCl, 0·1 M-NaCl, pH 7·0 at both 10 °C and 20 °C.

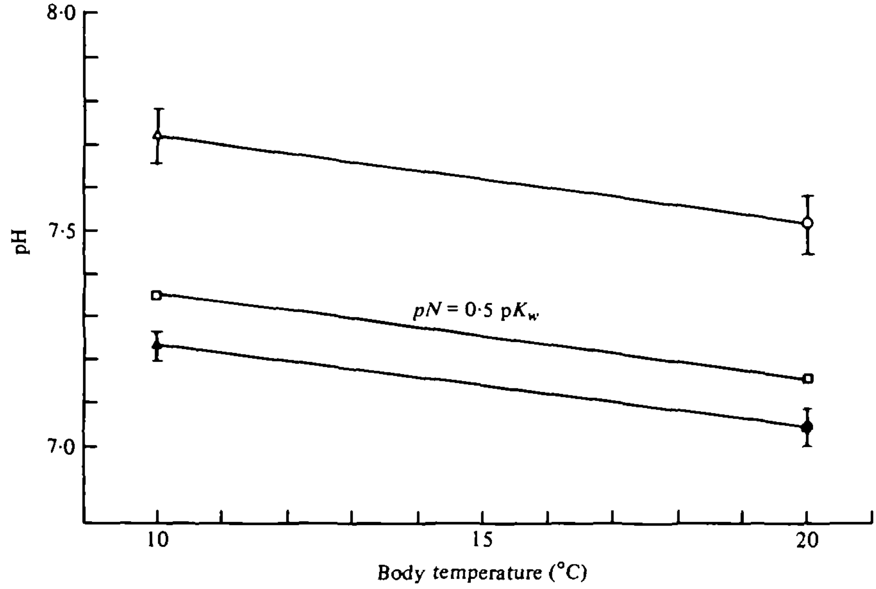

Fig. 5. Whole blood pH and intraerythrocytic pH of resting 10 °C- and 20 °C-acclimated blackfish, measured at their respective temperatures of acclimation: 10 °C-acclimated fish whole blood pH at 10 °C, △; 10 °C-acclimated fish intraerythrocyte pH at 10 °C, ▲; 20 °C-acclimated fish whole blood at 20 °C, ○; 20 °C-acclimated fish intraerythrocytic pH at 20 °C, ●. Neutral point of water (pN) as a function of water temperature, □. Experimental conditions (blood pH was measured on individual fish immediately following blood withdrawal): 20 °C fish, 20 individuals; 10 °C fish, 18 individuals. Each point represents the mean value, with the standard deviation indicated by vertical bars.

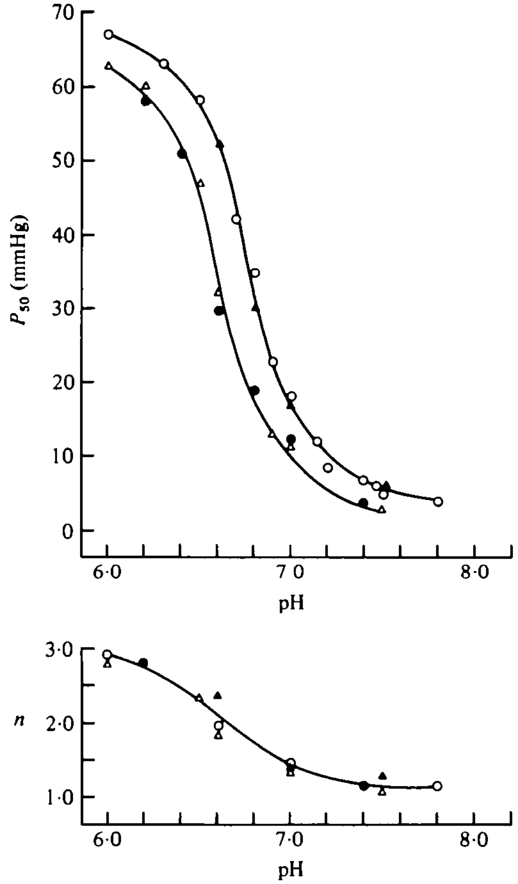

Fig. 6. Comparison of the effects of pH on the oxygen affinity (P_{50}), and Hill coefficient (*n*) of solutions of stripped haemoglobin from 10 °C- and 20 °C-acclimated blackfish, determined at 10 °C and 20 °C: 10 °C-acclimated fish at 10 °C, △; 20 °C-acclimated fish at 20 °C, ▲; 20 °C-acclimated fish at 10 °C, ●; 20 °C-acclimated fish at 20 °C, ○. Experimental conditions: equilibration P_{CO_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.

Effects of thermal acclimation on interactions between the oxygen-binding properties of whole blood and haemoglobin solutions, and pH *and carbon dioxide*

Although blood pH changes with assay temperature, similar values were obtained when blood samples from either acclimation group were compared at the same assay temperature (Table 1). Values for whole blood pH and intraerythrocytic pH were lower at 20 °C than at 10 °C, with the change in pH/°C (Δ pH/Δ °C) of −0·02 in each case (Fig. 5). These changes in pH with temperature parallel the neutral point of water, indicating that blackfish, like other poikilotherms, regulate relative alkalinity (OH^-/H^+) rather than hydrogen ion concentration *per se* (Rahn, 1967).

The relationship between mixed arterial-venous blood pH, P_{CO_2}, plasma bicarbonate and acclimation temperature are given in Table 1. While blood pH and plasma bicarbonate are lower at 20 °C, there is no significant difference in the measured P_{CO_2} the mixed arterial-venous blood.

The effects of pH on the oxygen affinity and *n* values of solutions of stripped haemoglobin from the two acclimation groups are shown in Fig. 6.

As reported previously (Dobson & Baldwin, 1982), the stripped haemoglobin solutions did not show any oxygen-linked binding of carbon dioxide.

DISCUSSION

Changes in the whole blood oxygen equilibrium curve with thermal acclimation

Blackfish acclimated at 20 °C display whole blood oxygen equilibrium curves shifted to the right of curves obtained for 10 °C-acclimated animals, when assayed at either 10 °C or 20 °C (Fig. 1). This change in whole blood oxygen affinity with acclimation temperature might arise in several ways. Thermal acclimation could change the relative proportions of various molecular forms of haemoglobin that differ in oxygen-binding properties. It could also alter interactions between haemoglobin and intraerythrocytic modulators such as organic phosphates, pH and carbon dioxide, that regulate oxygen-binding properties.

In this study, the first possibility can be dismissed on the following grounds: (1) oxygen equilibrium curves obtained for solutions of stripped haemoglobin prepared from blood of 10 °C- and 20 °C-acclimated fish did not show the differences observed with whole blood (Fig. 3); (2) electrophoretic analysis revealed that thermal acclimation did not alter the relative proportions or mobilities of the five molecular forms of haemoglobin present.

As the major intraerythrocytic regulators of haemoglobin oxygen affinity in the 20 °C-acclimated blackfish are known to be nucleoside triphosphates and pH (Dobson & Baldwin, 1982), particular attention was given to the effects of thermal acclimation on interactions of these two regulators with haemoglobin.

The concentrations of total nucleoside triphosphates was higher in erythrocytes of 20 °C-acclimated fish, whereas the ATP:GTP ratio remained at 5:1 in both acclimation groups. Since organic phosphates interact with haemoglobin in the ratio of 1 mole per mole of tetrameric haemoglobin (Benesch & Benesch, 1969), it is most useful to express the nucleoside triphosphate levels in the red cell as molar ratios relative to haemoglobin. The mean NTP:Hb_4 values were 1·32 and 1·68 respectively for the 10 °C- and 20 °C-acclimated fish. This striking difference in molar ratio arises from both higher concentrations of nucleoside triphosphates and lower concentrations of haemoglobin in the blood of 20 °C-acclimated fish (Table 1).

The effects of NTP:Hb_4 molar ratios on oxygen affinity are indistinguishable for stripped haemoglobin solutions prepared from 10 °C- and 20 °C-acclimated fish (Fig. 4). These curves also show that the changes in molar ratios associated with thermal acclimation occur in the range that has most effect on haemoglobin oxygen affinity, and would act to reduce whole blood oxygen affinity in the 20 °C-acclimated fish.

It appears that the changes in pH are not involved in producing the differences in whole blood oxygen affinity associated with thermal acclimation, as similar whole blood and intraerythrocytic pH values were obtained for each acclimation group when compared at the same assay temperature. In addition, the effects of pH on the stripped haemoglobin solutions from the two acclimation groups were the same (Fig. 6).

Functional significance of changes in blood oxygen transport properties in fish accompanying thermal acclimation

The blood oxygen transport system in water-breathing fish represents a compromise between maintaining a sufficiently high oxygen affinity for loading oxygen from an environment that is oxygen deficient relative to air, and maintaining a sufficiently low affinity to permit delivery of adequate amounts of oxygen at the tensions encountered in tissue capillaries. As changes in temperature can influence both environmental oxygen availability and oxygen-dependent metabolic rate functions, the oxygen-binding properties may be adjusted during thermal acclimation to provide the best compromise between oxygen uptake and oxygen delivery under the new thermal state. In this situation either an increase or a decrease in blood oxygen affinity accompanying thermal acclimation could be interpreted as adaptive.

When comparing the oxygen transport properties of whole blood from blackfish acclimated to 10 °C and 20 °C, the major difference observed was the reduced oxygen affinity in fish at the higher acclimation temperature. A lower-affinity blood is thought to enhance unloading of oxygen to the tissues at rest, or during and following active swimming. Presumably, for the blackfish this reduction in affinity at the higher acclimation temperature does not cause problems with loading at the gills, because of the high oxygen tensions (130–140 mmHg) normally encountered in its natural environment. The shift to a lower oxygen affinity could be considered adaptive if it was associated with elevated rates of oxygen-dependent metabolism at higher temperatures (Fry, 1957, 1971), but such metabolic rate data is not available for the blackfish.

Grigg (1969) found that the effects of thermal acclimation and acclimatization on the whole blood oxygen equilibrium curve of the bullhead catfish *Ictalurus nebulosus* were the opposite to those observed for the blackfish, with high temperature increasing blood oxygen affinity. This change was interpreted as adaptive in that it acted as a homeostatic mechanism tending to maintain similar P_{50} values at both high and low acclimation temperatures, thereby minimizing the effects of temperature on blood oxygen affinity. An alternative interpretation, which is more compatible with the changes in blood oxygen affinity observed for both *Ictalurus* and the blackfish, is as follows. The blood of *Ictalurus* has a very high oxygen affinity and low Bohr effect relative to blackfish, and these properties are considered adaptations to life in stagnant acidic waters with low oxygen content (Haws & Goodnight, 1962). With oxygen uptake at a premium in such an environment, and possibly placing severe restrictions on oxygen-dependent metabolism, increased blood oxygen affinity at high temperatures would aid oxygen loading at the gills at a time when the oxygen content of the water was at a minimum. Thus differences in the responses of blood oxygen affinity to thermal acclimation in these two species can be seen as reflecting underlying differences in the effects of temperature on both habitat oxygen availability and oxygen-dependent metabolic rates.

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