Astyanax scabripinnis (PISES: CHARACIDAE)
HEMOGLOBINS: STRUCTURE AND FUNCTION

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ABSTRACT
Electrophoretic patterns of hemoglobins, Root effect, Bohr effect in blood and stripped hemoglobin, Hb-O2 affinity GTP modulation of Astyanax scabripinnis (lambari), caught at three different altitudes in Ribeirão Grande, near Campos do Jordão (São Paulo), are described. All populations showed the same electrophoretic patterns: two cathodal components in starch gel. Normal Bohr effect values were found in these three populations both in blood ($\phi = -0.11$) and stripped hemoglobin ($\phi = -0.12$). Different blood O2 affinities collected in fish of these 3 populations were detected. GTP has a large influence on Hb-O2 binding properties in A. scabripinnis. Stripped hemoglobin shows small Root effect. The addition of triphosphated nucleotides increases this effect. GTP is more effective than ATP on enhancing Root effect. Oxygen availability in water can be the factor responsible for differences found in blood O2 affinity.

Key words: lambari, oxygen availability, organic phosphates, Bohr effect, altitude.

INTRODUCTION
Hemoglobins are particularly important in studies of fish adaptations because the hemoglobin molecule is poised between the metabolism of the organism and the environment and so it has to cope with both metabolic requirements and environmental constraints (Riggs, 1979).

The Astyanax scabripinnis individuals studied here, live in river headwater, and in mountains, forming separated populations that are able to migrate down, but very seldom to it.

Krogh & Leitch (1911) showed that blood respiratory properties could be related to availability of environmental oxygen. After this report, many authors showed that differences exist in
functional properties of hemoglobin in fishes from different environments.

The present paper compares functional properties of unfractionated blood, hemoglobin electrophoretic pattern, functional properties of stripped hemoglobin and the influence of organic phosphates in O2 affinities, from three populations living at three different altitudes of *Astyanax scabripinnis* in Campos do Jordão, SP, Brazil.

**MATERIAL AND METHODS**

Seventy two *Astyanax scabripinnis* individuals caught at ponds located at three altitudes of Ribeirão Grande were analyzed: 29 specimens from 1,920 m, 26 from 1,800 m and 17 from 700 m.

Blood was obtained by caudal puncture with heparinized syringes. While still in the field just after bleeding a suspension of unfractionated blood and yeast in a buffered solution (bis-tris and tris 0.1 M) was placed in a YSI Oxygen Monitor (model 53) and the O2 consumption was plotted by a recorder. Oxygen dissociation was determined at 20°C as described by Johansen et al. (1978a). The Bohr factor (\(\Delta\log P50/\Delta pH\)) was calculated from oxygen dissociation curves obtained at different pH values.

The red blood cells were washed 3 times with 0.9% NaCl, and then tris-EDTA 0.015 M, pH 8.0 was added to the packed erythrocytes in a 2:1 ratio. Hemolysis was achieved by freezing and thawing three times. To free the hemolysate of cellular debris, the sample was centrifuged at 12,000 \(g\) for 15 min at 4°C.

The hemoglobin solution was frozen at –20°C for further analysis in the laboratory.

In order to check if some variation was present in the samples, electrophoresis of individual hemolysates was carried out on starch gel as described by Smithies (1955, 1959) and modified by Val et al. (1981).

For spectrophotometric oxygen equilibria and Root effect experiments, the supernatant was then stripped of salt and organic phosphates by passage through a 2.5 cm x 30 cm column of Sephadex G-25 resin equilibrated in 0.1 mM Tris-HCl, pH 8.0. Methemoglobin, when present, was reduced with dithionite in a Sephadex G-25 column according to Schwantes et al. (1976), modified in the following way: the sample was applied only after no dithionite solution remained at the top of the resin; due to change in the sample colors, reduction and reoxygenation could be accompanied visually as the sample passes through the column.

Oxygen equilibria of stripped hemoglobin were carried out at 20°C by the spectrophotometric method described by Riggs & Wolbach (1956). The effect of GTP was measured by addition of appropriate volumes of solutions of ATP disodium salt to stripped hemoglobin solutions. GTP was tested in a molar ratio of 1:8 (Hb: NTP). The P50, Bohr effect and \(n\) values (Hill plot) were interpolated from linearized logarithmic plots of the O2 equilibrium data.

The Root effect was performed as described by Farmer et al. (1979) and the data were divided into four arbitrary categories based on approximate percentages: 0, less than 10% deoxygenation; +, 10%-20% deoxygenation; ++, 20%-40% deoxygenation; ++++, more than 40% deoxygenation. The amount of GTP and ATP used in these experiments was calculated as described above for GTP.

**RESULTS**

The hemolysates from several adult individuals were characterized electrophoretically. The analysis of 20 specimens (not all samples could be submitted to electrophoresis due to the very low amount of blood obtained) showed two hemoglobins components, one major and another minor band. This pattern was constant from one individual to another (Fig. 1).

Oxygen equilibria determined from unfractionated blood samples of *Astyanax scabripinnis* are shown in Fig. 2. The results obtained from blood for *A. scabrippinis* at the three studied altitudes showed normal Bohr effect, with increasing O2 affinity in more alkaline pHs. Little difference in oxygen affinities from blood samples from fish caught in any of these three altitudes were found. However the higher the altitude, the lower the affinity (Table 1).

The oxygen equilibria data on stripped hemoglobin, Bohr effect value was \(\phi = -0.10\) and GTP presence increased slightly this effect, resulting in \(\phi = -0.14\) (Fig. 3). Cooperativity values (\(n\)) for the *Astyanax scabripinnis* were approximately 1.0, showing low or no cooperativity, with or without GTP.
The Root effect analysis shows that ATP also modulates negatively Hb-O₂ affinity, but at a lower intensity than GTP (Table 2).

DISCUSSION

Electrophoresis is usually used in structural pattern characterizations of fish hemoglobins, i.e. number of components and relative concentrations. The support and system of buffers used varies and the choice of electrophoretic migration media depends on resolution power, costs, and speed, among other factors.

In this work, we used starch gel as a migration media for determining species electrophoretic patterns due to excellent resolution of this support for fish hemoglobin. Beside the samples obtained at the sites mentioned here, A. scabripinnis hemoglobin from other sites in the Campos do Jordão area were submitted to electrophoresis and showed an identical pattern. So this species is probably monomorphic for hemoglobin.

For the analyzed species, we observed that GTP decreases Hb-O₂ affinity showing that this phosphate acts negatively on Hb-O₂ affinity, and confirming the fact that GTP is the main modulator in fresh water fishes. These phosphates exert the greatest effect on P50 at 10 mM concentration and GTP seems to depress oxygen affinities more strongly than ATP at the same concentration (seen in Root effect experiments). The Root effect analysis shows that ATP also modulates negatively Hb-O₂ affinity, but less intensely than GTP does.

According to Weber et al. (1979), the concentration of GTP decreases more than ATP during hypoxia. Probably this happens with Astyanax scabripinnis too, since GTP is more effective than ATP as a negative modulator.

Studies of blood functional properties can be of the great value in understanding fish habitat and habits (Wood & Johansen, 1972; Powers, 1974; Greaney & Powers, 1978; Val et al., 1986). Many researches have demonstrated that fish species living in low oxygen concentration sites show greater blood affinity to this gas than those living in well oxygenated sites (Johansen et al., 1978a,b; Powers et al., 1979a,b; Powers, 1985; Val et al., 1986, 1990).

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### TABLE 1
Unfractioned blood, Hb functional properties, temperature and O₂ available in water at three altitudes.

<table>
<thead>
<tr>
<th></th>
<th>700 m</th>
<th>1,800 m</th>
<th>1,920 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractioned blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bohr effect</td>
<td>-0.11</td>
<td>-0.10</td>
<td>-0.11</td>
</tr>
<tr>
<td>Log P50 (pH 7.0)</td>
<td>1.12</td>
<td>1.2</td>
<td>1.31</td>
</tr>
<tr>
<td>Water temperature</td>
<td>21</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Oxygen available (mg/L)</td>
<td>9.5</td>
<td>10.2</td>
<td>10.4</td>
</tr>
</tbody>
</table>
Fig. 2 — Hemoglobin oxygen equilibria in blood: P50 X pH from Astyanax scabripinnis at 20°C. A. 1,920 m, B. 1,800 m, and C. 700 m.

**TABLE 2**

The Root effect in the presence and absence of phosphates (ATP and GTP).

<table>
<thead>
<tr>
<th>Hb</th>
<th>% deoxygenation</th>
<th>Farmer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stripped</td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>Stripped + ATP</td>
<td>22</td>
<td>++</td>
</tr>
<tr>
<td>Stripped + GTP</td>
<td>34</td>
<td>++</td>
</tr>
</tbody>
</table>

Fig. 3 — The pH effect on oxygen affinity of stripped hemoglobin from A. scabripinnis in the presence and absence of GTP at 20°C.
The data obtained on oxygen affinities and their pH dependence from unfractionated blood of *Astyanax scabripinnis* in three studied altitudes showed normal Bohr effect. This is positive for these fishes, since it facilitates O₂ release at the tissular level and uptake by the gills. However, the data obtained on blood-O₂ affinity shows slight differences depending on the altitude at which the sample was obtained (Table 1).

The different O₂ affinities in individuals caught at the three altitudes, can be due to oxygen availability and/or water temperature. At low altitudes (700 m) the water is warmer and oxygen concentration lower than at the others (1,800 and 1,920 m) (Table 1).

The hemoglobin Hb-O₂ affinity of vertebrates is regulated by allosteric effectors, such as chlorides (Cl⁻), protons (H⁺), carbon dioxide (CO₂), and several types of organic phosphates, besides temperature and salinity (reviewed by Weber & Jansen, 1988). In general, the increase of any of these factors or physical parameters within erythrocytes causes a drop in Hb-O₂ affinity.

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REFERENCES


