RESPIRATORY CHARACTERISTICS OF *Hoplosternum littorale* (SILURIFORMES, CALLICHTHYIDAE).

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ABSTRACT – Hematological parameters, intraerythrocytic phosphates, hemoglobin, and whole blood Bohr effect of the South American armored catfish *Hoplosternum littorale* were studied during different seasons of the year. In addition, the degree of dependence on air breathing was determined for this species. The hematological parameters presented seasonal variations, which were not correlated to oxygen, temperature, and water level oscillations. Five anodic hemoglobin fractions were detected in starch gel electrophoresis. In addition to ATP, GTP and Fe-GTP being detected, 2,3-DPG was also detected in red blood cells of *H. littorale*. The latter is an intraerythrocytic phosphate characteristic to red blood cells of mammalians. The increased production of 2,3-DPG could be associated with decreasing Hb-O_2 affinity and both features could be related to environmental temperature increase. Whole blood Bohr effect was influenced by water temperature. This study confirms *H. littorale* to be continuous and not obligate air breather, under all dissolved oxygen level conditions.

Key-words: Adaptation, air-breathing behavior, hematology, intraerythrocytic phosphate, blood Bohr effect.

Características Respiratórias de *Hoplosternum littorale* (Siluriformes, Callichthyidae).

RESUMO – Os parâmetros hematológicos, os fosfatos intraeritrocitários, a hemoglobina e o efeito Bohr do sangue de *Hoplosternum littorale* foram estudados em diferentes períodos do ano. Além disso, foi determinado o grau de dependência da respiração aérea nesta espécie. Os parâmetros hematológicos apresentaram variações sazonais, as quais não foram relacionadas com as variações no oxigênio dissolvido, temperatura e nível de água. Além de ATP, GTP e Fe-GTP terem sido detectados nos eritrócitos de *H. littorale*, 2,3-DPG também foi detectado. Este último é um fosfato intraeritrocitário característico de células vermelhas de mamíferos. O efeito deste composto sobre a afinidade da Hb-O_2 pode estar relacionado com o aumento da temperatura. O efeito Bohr do sangue foi influenciado pela temperatura da água. Este estudo confirma que *H. littorale* é um respirador aéreo facultativo que utiliza a respiração aérea mesmo em condições normóxicas.

Palavras-chave: Adaptação, comportamento da respiração aérea, hematologia, fosfatos intraeritrocitários, efeito Bohr do sangue.

INTRODUCTION

The Amazon region presents a complex and extensive aquatic ecosystem resulting from geological, physical and biological characteristics, such as the seasonal water level oscillations. Aquatic organisms, mainly fish, have developed adaptive mechanisms for facing these fluctuations. These mechanisms include air-breathing, aquatic surface respiration, oxygen transfer adjustments, and metabolism modification (Almeida-Val *et al.*, 1993; Val, 1995; Val & Almeida-Val, 1995).

Hematological parameters (hematocrit, hemoglobin concentration, and red blood cell number), intraerythrocytic phosphates, and the proportion between hemoglobin fractions are some of the parameters ad-

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justed for improving the oxygen transfer. Amazonian fish adjust these parameters when they are exposed to low oxygen availability and/or changes on environmental conditions associated with dry and rainy seasons (Monteiro *et al.*, 1987; Val *et al.*, 1990; 1992).

Many studies have been carried out on the regulatory importance of intraerythrocytic phosphates in vertebrates during the oxygen transport (Bartlett, 1978a; 1980; Val *et al.*, 1992a,b; Wilhelm Filho *et al.*, 1992; Marcon & Val, 1996). ATP and GTP are the most common organic phosphates in most fish, and their concentration variation may be influenced by environmental changes. In addition, other organic phosphates, such as IP_2, IP_5 and 2,3-DPG, were detected in this group's red blood cells.

This paper describes the respiratory mode and some respiratory parameters of *Hoplosternum littorale,* commonly know in Brazil as tamoatá. It can be found in stagnant, poor in oxygen shallow waters, such as the Paraguayan chaco, Guyana savanna, Venezuelan llanos and Amazonian flooded forest (várzea or igapó). *H. littorale*, as all other previously investigated Callichthyidae members, is an air-breather which uses the posterior intestine as an accessory respiratory organ.

MATERIALS AND METHODS

The armored catfish *Hoplosternum littorale* were captured in the Solimões river near the island of Marchantaria (3°15'S,60°00'W), during low (September, November, and January) and high (March, June and August) water period.

Blood samples were taken from the caudal vein by using heparinized syringes immediately upon capture. Hematocrit values were measure by the microhematocrit method, hemoglobin concentrations by cyanomethemoglobin method, and the red blood cells (RBC) were counted by using a Neubauer chamber. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined by using classical equations.

The water-soluble phosphates were extracted with 0.6 M perchloric acid from a pool of red blood cells washed in saline solution. The extracts were neutralized with 6 M KOH by using phenol red as indicator and frozen (-20°C). The extracts were chromatographed within a month according to the method described by Bartlett (1959) by using a 28x1cm column of DOWEX 1x8, formate form (200-400 mesh). The presence of 2,3-DPG was confirmed by the spectrophotometric method according to Dische & Borenfreund (1951).

Blood oxygen affinity was measured in the field by using a YSI model 53 biological oxygen monitor as described by Colman & Longmuir (1963). In a monitor chamber, three drops of fresh whole blood were added to an aerated suspension of yeast in 0.1 M Tris (tris-hydroxymethyl-aminomethane) or Bis-Tris (bis 2-hydroxyethyl-iminotris-hydroxymethyl-methane) chloride

buffers. Six different pH values (pH 6.2; 6.8; 7.4; 8.0; 8.6; 9.2) were used. The rate of decrease in oxygen concentration was recorded on a Pharmacia chart recorder.

The hemoglobin solutions used for electrophoretic analyses were obtained by removing the plasma and washing the red blood cells three times with saline solution (0.9% NaCl). The red blood cells were lysed in 1 volume of 5 mM Tris, pH 8.0, frozen and stored at -20°C. Two drops of 0.1 M KCN pH 8.0 were added to the samples and stroma was discarded by centrifuging at 20,000g for 20 min at 4 °C in a Sorvall RC-5B centrifuge. The hemoglobin electrophoretic patterns were determined by agar-starch coated slides as described by Araujo *et al.* (1970) and Machado (1973) and by starch gel according to Smithies (1955) using corn starch for both systems. Gel buffer systems were 0.036 M Tris-borate-EDTA, pH 8.6 and 0.35 M borate buffer, pH 8.6 was used in the electrode chambers. The starch gels were sliced and stained with Amido Black 10B (total proteins) and benzidine (peroxidasic activity).

Six animals were held in aquaria (31.5x57x30 cm) with normoxic water (7 mg/L) without access to air, and another six were held with access to air in hypoxic water (2 mg/L) at 25-27 °C for 9 days, for examining the respiratory mode of *H. littorale*. Two other groups of six fish were maintained for two days in aquaria at the same temperature and oxygen as described above, for testing air and branchial breathing frequency. Air-breathing frequency was measured on fish in normoxia and hypoxia conditions with access to the surface, for one hour and branchial breathing frequency was measured for one minute every 8 hours. Twenty-four hours before the experiment, all test fish were transferred to the aquaria and maintained in normoxia without food, at the same thermal acclimation temperature (26 ± 1°C). In hypoxic conditions, 12 h prior to the experiment, the oxygen tensions were reduced gradually from 7 to 2 mg/L. Water oxygen concentrations were adjusted during the experiment, by using the bubbling air or N_2 and their levels were estimated with a YSI dissolved oxygen electrode/thermistor.

Data are shown as mean ± SD. The F-test was used for checking the homogeneity of variance. One-way ANOVA followed by a post-hoc Schefé-test was used for determining the statistical significance of data ($p<0.05$). Relationships between different periods were determined through linear regression analysis.

RESULTS

Respiratory mode

All fish tested survived 9 days with or without access to the surface in both hypoxic (2 mg/L) and normoxic water (7mg/L). We observed that fish without access to the surface attempted reach the surface persistently, increased their brachial breathing frequency and activity level when compared to fish with access to air. The results of the comparisons between air and branchial breathing frequency on *H. littorale* when exposed to normoxia or hypoxia are summarized on Table 1. Lower air breathing

and higher branchial breathing frequencies were observed in animals exposed to normoxia.

Seasonal studies

Environmental aspects: Water level of the river, dissolved oxygen concentrations and temperature 10 cm deep are shown in Figure 1. The highest water level was detected in June and the lowest in November. Dissolved oxygen concentration was extremely low during March (0.15 mg/L) and high during August (11.0 mg/L). The highest temperature was in September (35.5 °C) and the lowest in June (25 °C).

Hematology: Hematocrit (Ht), hemoglobin concentration ([Hb]), red blood cell number (RBC), MCV, MCH and MCHC mean values are summarized in Table 2. There was no

Table 1. Aerial and branchial breathing frequency of *H. littorale* (n = 6) exposed to normoxia and hypoxia. Values are mean ± SD. *n* = number of fish assayed; Wt = fish weights.

Treatment	n	Wt (g)	Aerial (breaths/h)	Branchial (operc. beat/min)
Normoxia	6	66.7±5.2	2.1±0.5*	155.8±4.4*
Hypoxia	6	63.5±6.3	8.5±0.25	131.8±8.3

* Significant difference ($p<0.05$).

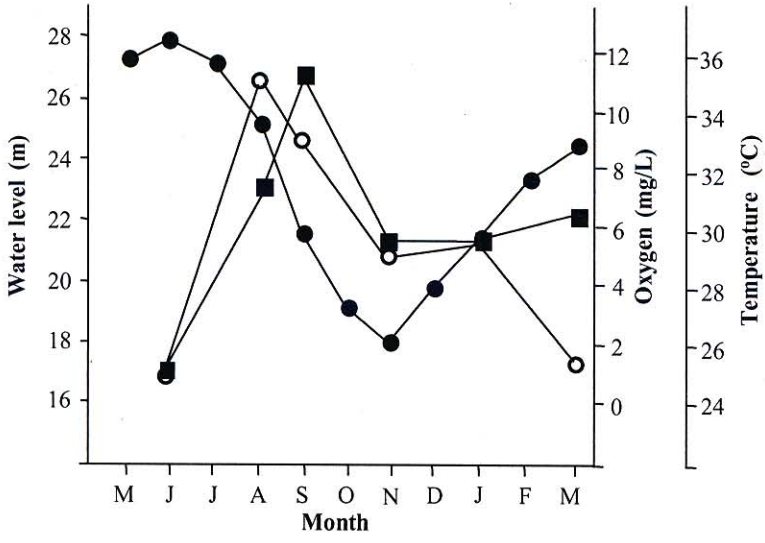

Figure 1. Water level (●), oxygen dissolved (○) and temperature (■) at the depth of 10 cm during all collecting periods. The water level data were obtained at Capitania dos Portos de Manaus and are relative to the Negro River water levels.

significant correlation between these parameters and the environmental variables (O_2, temperature and water level). However, Ht, [Hb], MCHC, and RBC mean values, were significantly higher in November, when the water level was low, and lower in March, when the water level was high.

Intraerythrocytic phosphate: intraerythrocytic phosphate chromatographic profiles for *H. littorale*, are shown in figure 2. ATP, GTP, Fe-GTP and 2,3-DPG were the main organic phosphates detected in the red blood cells of these fish. The concentrations of phosphate compounds detected in

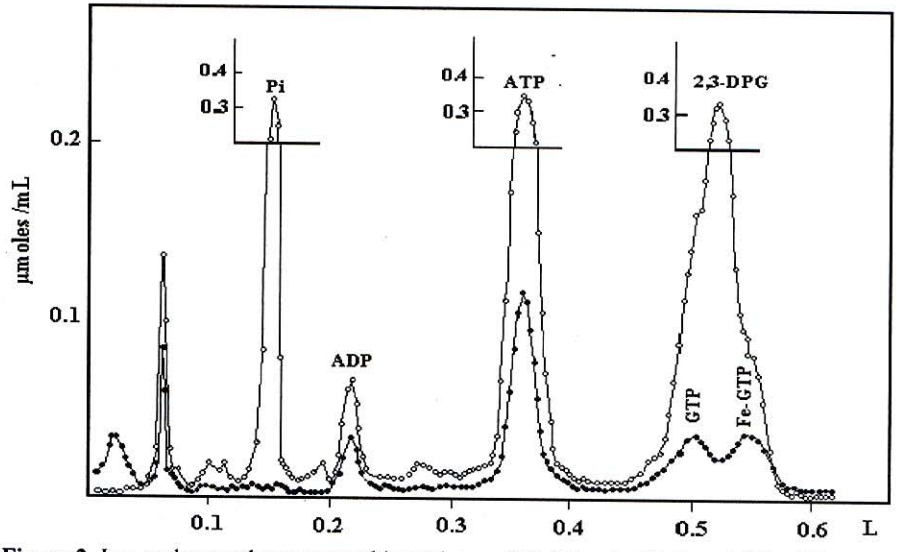

Figure 2. Ion-exchange chromatographic analyses of *H. littorale*. Elution: 0.8 L of linear gradient of 0-5 M ammonium formate buffer. All concentrations are expressed as µmol/mL of eluete. =●, optical density at 260 nm, calculated as µmol of adenine; O, total phosphorus.

Table 2. Hematological parameters of *H. littorale* specimens in different months. Values are mean ± SD. *n* = number of fish assayed. Different letters represent significant differences between parameters ($p < 0.05$).

	June	August	September	November	January	March
Ht (%)	38.5±1.2^{a}	39.1±0.7^{a}	42.1±0.4^{b}	43.7±0.9^{c}	38.8±0.6^{a}	37.2±0.6^{a}
Hb (g%)	12.5±0.5^{b}	12.9±0.3^{b}	13.7±0.1^{c}	16.1±0.3^{d}	12.6±0.2^{b}	11.1±0.4^{a}
RBC (x106)	1.9±0.1^{b}	1.9±0.06^{a,b}	2.3±0.08^{c,d}	2.5±0.06^{d}	1.8±0.04^{a}	2.3±0.1^{c}
MCV (µm3)	202.7±11.1^{c}	212.1±7.8^{c,d}	198.9±6.3^{b,c}	184.0±5.6^{a,b}	225.1±4.7^{d}	173.4±5.9^{a}
MCH (pg)	64.2±2.3^{b,c}	69.3±2.8^{c,d}	61.3±2.2^{b}	67.4±2.2^{c}	74.1±2.1d	51.6±2.1^{a}
MCHC (%)	32.6±1.5^{b}	32.6±0.5^{b}	32.9±0.3^{b}	36.7±0.5^{c}	32.7±0.6b	29.8±0.8^{a}
n	20	29	70	50	67	50

the cromatographic analyses are shown in Table 3. Only 2,3-DPG concentrations and 2,3-DPG/Hb ratios were correlated according to the water temperature (Fig. 3). There were no detectable inositol polyphosphates in any of the samples analyzed.

Whole blood Bohr effect: The Bohr shifts obtained from *H. littorale* blood oxygen equilibrium showed a normal Bohr effect ($\phi = \Delta \log P_{50}/\Delta pH$= oxygen pressure necessary to sature 50% of Hb solutions) during the analyzed situations (Tab. 4). The whole blood Bohr effect values oscillated according to water temperature (Fig. 4A), but were not correlated to dissolved oxygen and water levels. The highest Bohr effect was obtained in June, when water level was high. The whole blood Bohr effect values were correlated to 2,3-DPG and 2,3-DPG/Hb ratios (Figs. 4B, 4C).

Electrophoretic studies: *H. littorale* hemoglobin patterns, showed five and two anodic hemoglobin fractions detected on starch and agar-starch slides gel electrophoresis, respectively, during the dry and rainy seasons (Fig. 5). The largest number of hemoglobin

Table 3. Main intraerythrocytic phosphates concentrations of *H. littorale* during collectiong periods, expressed as mmol/mL of packed red blood cells. Main phosphates to hemoglobin molar ratios are expressed as mM NTP/mM Hb. NTP= (ATP + GTP); TP= total phosphates (ADP + ATP + GTP + Fe-GTP + 2,3-DPG).

	June	September	November	January	March
Pi	0.803	2.354	2.168	2.730	2.350
ADP	0.118	0.370	0.431	0.366	0.457
ATP	0.498	1.547	1.772	2.072	2.561
GTP	1.190	1.681	3.514	9.501	8.376
Fe-GTP	0.556	4.431	3.723	4.988	10.276
2,3-DPG	0.563	1.853	1.034	1.016	1.082
NTP	1.688	3.228	5.286	11.573	10.937
ATP/GTP	0.418	0.920	0.504	0.218	0.306
2,3-DPG/NTP	0.334	0.574	0.196	0.088	0.099
TP	2.925	9.882	10.474	17.943	22.752
ATP/PT	0.177	0.157	0.169	0.115	0.102
GTP/PT	0.424	0.170	0.335	0.530	0.334
2,3-DPG/PT	0.201	0.188	0.099	0.057	0.043
ATP/Hb	0.098	0.303	0.311	0.408	0.554
GTP/Hb	0.235	0.330	0.617	1.872	1.813
Fe-GTP/Hb	0.110	0.869	0.654	0.983	2.225
2,3-DPG/Hb	0.111	0.363	0.182	0.200	0.234

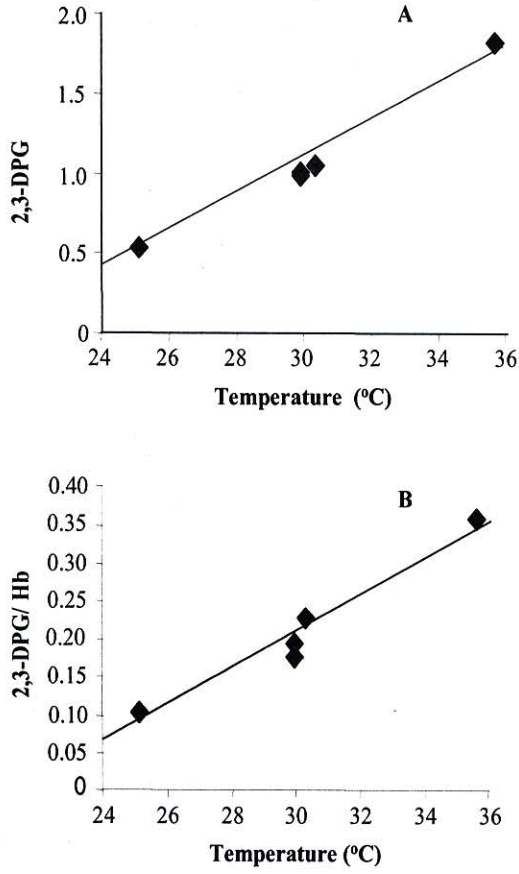

Figure 3. Relationships between 2,3-DPG expressed as μM/mL of packed RBC and water temperature (A, r=0.99) and between molar ratio 2,3-DPG/Hb expressed as mM NTP/mM Hb and water temperature for *H. littorale* (B, r=0.98).

Table 4. Bohr effect and log P_{50} for *H. littorale* during the collecting periods. *n* = number of fish assayed.

	n	Borh effect(ϕ)	LogP50pH 7.0
June	6	-0.110	0.880
August	6	-0.135	0.803
September	8	-0.174	0.825
November	6	-0.150	0.856
January	7	-0.157	0.886
March	8	-0.160	0.849

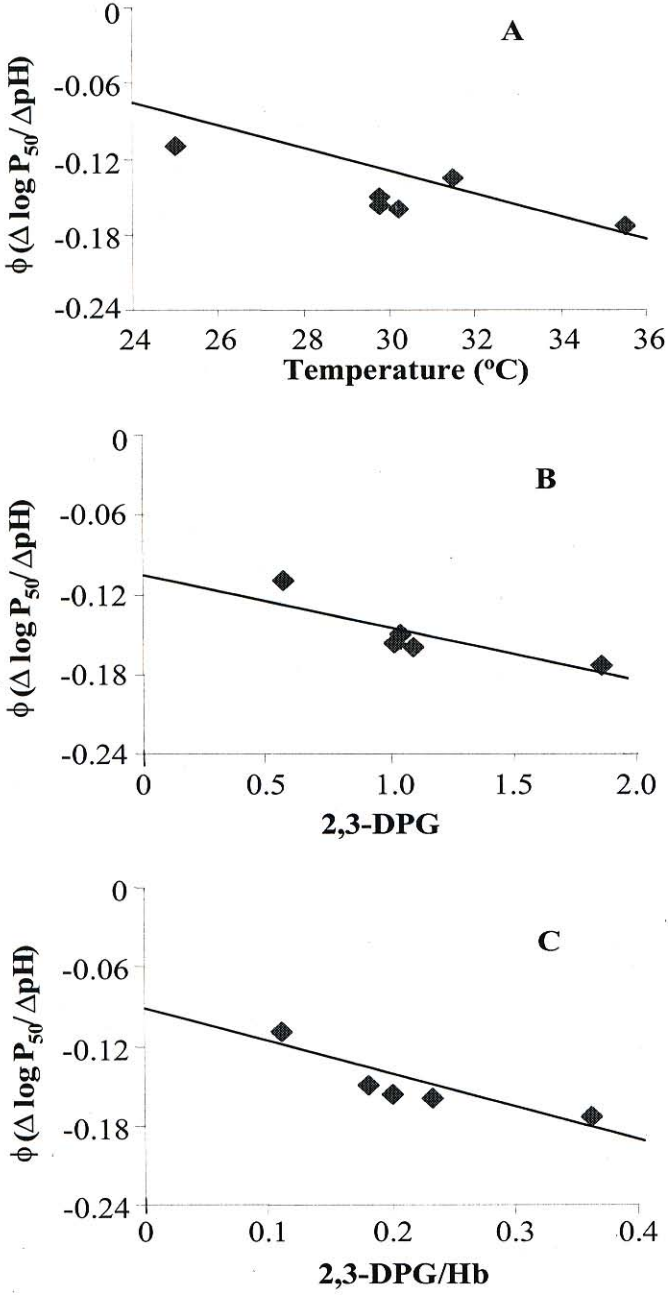

Figure 4. Relationships between ϕ ($\Delta \log P_{50}/\Delta pH$) and temperature (A, r=0.83), ϕ and 2,3-DPG expressed as μM/mL of packed RBC (B, r=0.86) and the ratio 2,3-DPG/Hb (C, r=0.87) for *H. littorale*.

fractions found in the starch gel was probably due to gel resolution power differences.

DISCUSSION

This study has confirmed that *Hoplosternum. littorale*, as well as all

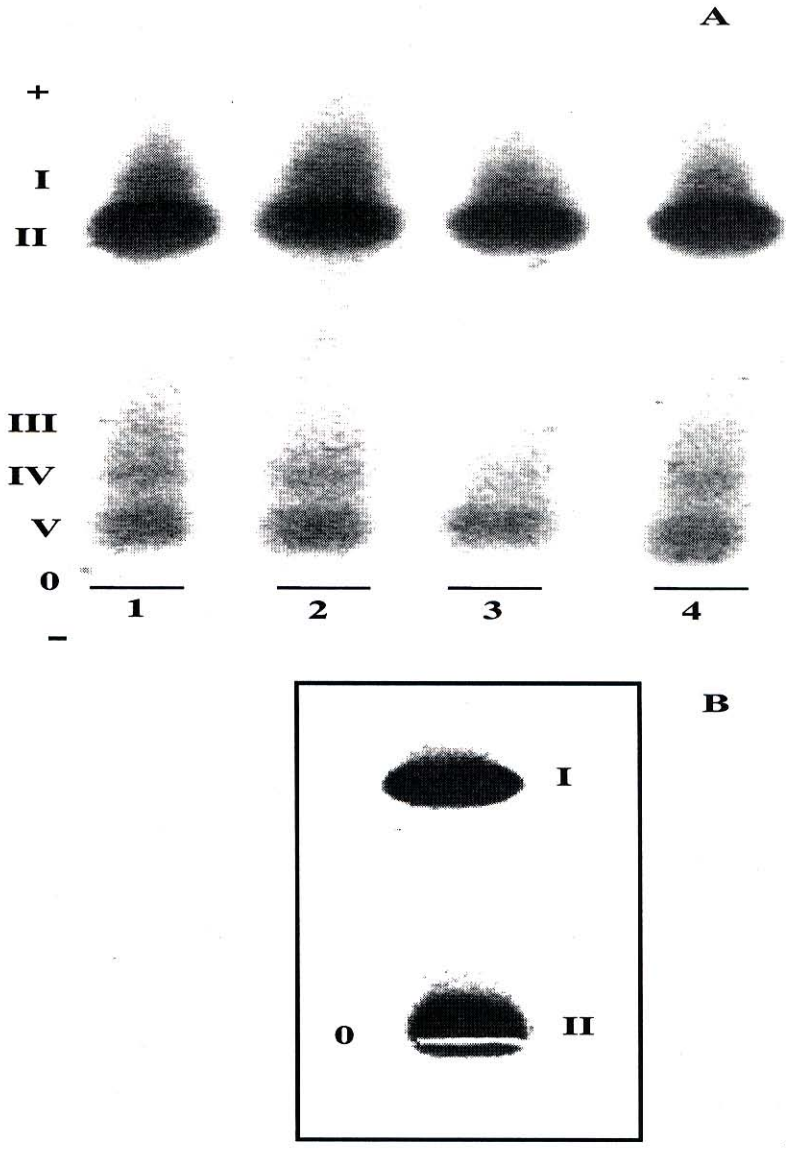

Figure 5. Hemoglobin electrophoretic patterns *of H. littorale* on starch gel (A) and agar-starch coated slices (B).

previously investigated members of Callichthidae, is a continuous but not obligate air breather. Although this species breathes continuously, both aerial and aquatically, the contribution of the two modes varies according to oxygen levels. We have shown that *H. littorale* presents oxygen consumption (VO_2) lower in hypoxic than in normoxic water (Affonso, 1999). In response to aquatic hypoxia, *H. littorale* increases and lowers its air and branchial breathing frequencies respectively. These results, coupled with low VO_2 in hypoxia, suggest air breathing is an important adaptive strategy to *H. littorale.*

The regularly high values for hematocrit (40%), hemoglobin concentration (12g%), and number of circulating red blood cells ($2.5x10^6$), observed in *H. littorale,* at different seasons of the year, certainly contribute to maintain oxygen transfer to the tissues in natural environmental conditions. This result is corroborating with Val & Almeida-Val (1995) in a review about physiological and biochemical features of the Amazonian fishes.

In general, the hematological parameters give an excellent picture of the processes occurring in the organism under the influence of environmental changes. Although none of the analyzed *H. littorale* hematological parameters showed any correlation with water temperature, oxygen concentration and level oscillation, they presented significant seasonal variations. An example of those was the higher hematological parameters mean values observed in November (low water level). (We believe that under these conditions, fish being confined within small lakes with lesser and lesser food resources, as well as higher and higher rate of predation, become highly stressed. Conversely, all mean hematological parameter values but for RBC, were lower in March (high water level). By the observations made on the collecting site, one may suppose the presence of hydrogen sulfide (H_2S), detected by its strong odor, might have been the cause of such anemia. This compound is highly toxic to aerobic organisms (Bagarinao, 1992), and it is probably one of the factors causing fish mortality in varzea lakes (Affonso & Waichman, *in press*).

Many fish species exhibit multiple hemoglobin fractions (Fyhn *et al.*, 1979; Galdames-Porto *et al.*, 1982; Val *et al.*, 1992a). These have been considered a fish adaptive strategy to environmental flutuations, since it was showed that in many fish species they present different functional properties (Weber *et al.*, 1976; Garlick *et al.*, 1979; Val, 1993).

Perez (1980) and Garlick *et al.* (1979) detected two Hb fractions with different functional properties in *H. littorale.* In the present study, even though *H. littorale* shows two anodic hemoglobin fractions on agar-starch slide gel, the five Hb components detected on starch gel should better reflect the hemoglobin heterogeneity in this species, because starch gel probably renders higher resolution than the mixture of agar-starch. The study of Wilhem Filho & Weber (1983) con-

cerning this subject revealed that *Callichthys callichthys*, a related callichthyid species, also possesses 5 Hb components in starch gel eletrophoresis and only 2 in acetate slides, corroborates the interference of resolution capacity of different substrates. In the present work, the multiple hemoglobin *H. littorale* did not show any correlation with environmental variations in the different collecting periods. However, the presence of these five different hemoglobin fractions may be an important factor showing their concentration regulation according to environmental oscillations.

The nature and quantity of organic phosphates in fish species red cells are not as well studied as in the higher vertebrates. ATP and GTP have been frequently detected in the erythrocytes of fish acting as negative modulators of the Hb-O_2 affinity. Other characteristic organic phosphates of the higher vertebrates such as 2,3-DPG in mammalian and IP_5 in birds, have been detected in red blood cells of some fish species (Gillen & Riggs, 1971; Isaacks *et al.*, 1977; 1978; Bartlett, 1978b; Val *et al.*, 1992b).

In fish, 2,3-DPG was detected only in *Cichlasoma cyanoguttatum*, *Liposarcus* sp (=*Pterygoplichthys* sp) and now in *H. littorale*. In the former species, it is not known if the presence of this phosphate is in enough quantity to act as oxygen transport controller. Val *et al.* (1990) questioned its presence on *Liposarcus* blood.

H. littorale presented quantitative changes on 2,3-DPG during the five collecting trips. Although 2,3-DPG concentration variations and 2,3-DPG/Hb molar ratios were not correlated with water oxygen concentrations or level, they were positively correlated with temperature. By contrast, in some fish, the red organic phosphate concentrations decreased as temperature rose (Greaney & Powers, 1977; Houston & Koss, 1984; Nikinma, 1990). We have observed this fish does not adequately tolerate high temperatures (above 35°C) and needs to increase its air and branchial breathing frequency (Affonso, 1999). Since *H. littorale* moves around macrophytes from a drying lake into the river or into another lake during the dry season, it faces high temperatures. We suggest that, if this species possesses an uncommon erythrocytic phosphate, such as 2,3-DPG, this compound may act as an important regulator of Hb-O_2 affinity at high temperatures. But it would be necessary to study its effect on acclimated *H. littorale* at various temperatures and oxygen concentrations in further investigations.

The remaining phosphate compounds, Fe-GTP, GTP and ATP, present quantitative variation, just as 2,3-DPG. Of these four organic phosphates detected, Fe-GTP showed higher concentration in all five collecting trips. Despite the high and variable amounts of Fe-GTP reported in the red blood cells of some fish species, the physiological role of this compound is still not known (Bartlett, 1980; Monteiro *et al.*, 1987; Val *et al.*, 1990).

The ATP/GTP ratio of *H. littorale* was lower than 1.0 during the

two study periods. Johansen *et al.* (1978), when studying this same species under aquatic and air-breathing respiration, obtained the same ATP/GTP ratio found in the present study. Therefore, the higher GTP than ATP concentrations in the red blood cells, is independent from the respiratory behavior of *H. littorale*

Although Fe-GTP, GTP and ATP have not showed any significant relation ($p<0.05$) to the three environmental parameters measured in the present work, the increase on the concentration of these phosphates coincided with the increase of the water level. It has been reported that water level oscillations cause big changes in the aquatic habitat, and consequently on the Amazonian fish biology (Val & Almeida-Val, 1995). The variation in the concentrations of these phosphates may be an adaptive response for facing any other stress factor due to these water-level fluctuations.

The positive correlation found in *H. littorale* between the whole blood Bohr effect values (ϕ) and the different water temperatures, corroborated with the results described by Perez (1980) when studying the same species. Therefore, the high blood Bohr effect found in *H. littorale*, when exposed to high temperatures may facilitate the release of O_2 into the tissues.

Previous studies have shown Bohr effect variations in fish to be directly influenced by organic phosphate concentrations (Weber *et al.*, 1979; Val *et al.*, 1986; Monteiro *et al.*, 1987). The 2,3-DPG: Hb/ϕ and 2,3-DPG/ϕ relations obtained for *H. littorale* showed the increases of 2,3-DPG and ϕ were related, which could be associated with the decreasing of Hb-O_2 affinity in higher temperatures.

All parameters investigated during the seasonal study (hematology, intraerythocytic phosphates, hemoglobin, and Bohr effect) did not present any correlation with dissolved O_2 variations. The ability of *H. littorale* to tolerate extreme hypoxic conditions in its natural environment may represent the air-breathing efficiency in this species, which certainly should maintain the "O_2 cascate".

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