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Air-breathing behavior and physiological responses to hypoxia and air exposure in the air-breathing loricariid fish, *Pterygoplichthys anisitsi*

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Abstract Hypoxic water and episodic air exposure are potentially life-threatening conditions that fish in tropical regions can face during the dry season. This study investigated the air-breathing behavior, oxygen consumption, and respiratory responses of the air-breathing (AB) armored catfish *Pterygoplichthys anisitsi*. The hematological parameters and oxygen-binding characteristics of whole blood and stripped hemoglobin and the intermediate metabolism of selected tissue in normoxia, different hypoxic conditions, and after air exposure were also examined. In normoxia, this species exhibited high activity at night and AB behavior (2–5 AB h^{-1}). The exposure to acute severe hypoxia elicited the AB behavior (4 AB h^{-1}) during the day. Under progressive hypoxia without access to the water surface, the fish were oxyregulators with a critical O_2 tension, calculated as the inspired water O_2 pressure, as 47 ± 2 mmHg. At water O_2 tensions lower than 40 mmHg, the fish exhibited continuous apnea behavior. The blood exhibited high capacity for transporting O_2, having a cathodic hemoglobin component with a high Hb–O_2 affinity. Under severe hypoxia, the fish used anaerobic metabolism to maintain metabolic rate. Air exposure revealed physiological and biochemical traits similar to those observed under normoxic conditions.

Keywords Respiratory physiology · Air-breathing fish · Oxygen transport cascade · Hypoxia · Air exposure

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Introduction

Environmental hypoxia is a natural phenomenon caused by the vertical stratification of the water column, low photosynthetic activity, low or stagnant water flow, and temperature and salinity fluctuations (Dejours 1981; Karim et al. 2003). Under hypoxic conditions, air-breathing (AB) fish satisfy their metabolic O_2 demands by acquiring O_2 directly from the air via specialized AB organs; this O_2 complements or fully replaces the O_2 normally obtained by the gills (Graham 1997). Modifications of ventilation (Hebert and Wells 2001; Alton et al. 2007), blood–O_2-binding affinity (Clementi et al. 1994; Weber et al. 2002), and

the rearrangement of metabolic pathways are responses that compensate for low environmental O_2 availability and are exhibited by water-breathing fish and by AB fish if access to the water surface is denied (van den Thillart and van Waarde 1985; van Hesswijk et al. 2005).

Oxygen uptake from the environment and the transport of O_2 to the tissues depend on convective and diffusive processes. The oxygen transport from the environment to the tissues (the "oxygen transport cascade") consists of four different steps that occur in series: (1) ventilation, (2) diffusion of O_2 across the gas exchange surface, (3) circulation, and (4) diffusion of O_2 into the cells (Wang and Hicks 2002), and the amount of O_2 carried per unit volume of blood depends on factors such as the O_2 tension, the number of erythrocytes, the amount of hemoglobin (Hb) in the red blood cells, and the Hb–O_2 affinity (Nikinmaa 1997). According to the concept of symmorphosis (Weibel et al. 1992), each of these steps needs to be optimized to guarantee efficient O_2 transport from the environment to the tissues. In AB fish, steps 3 and 4 are identical for gas exchange occurring in water or air, but step 1 and 2 may vary considerably mainly due to the diffusive capacities of the water- and air-breathing organs.

Compensation for the reduction in O_2 levels during aquatic hypoxia may be aided through biochemical as well as physiological adjustments. Therefore, organisms need to maximize O_2 uptake by biochemical adjustments (Hochachka and Somero 2002). Such biochemical adjustments are observed mainly in water-breathing species that are highly tolerant to hypoxia; the fish combine metabolic suppression and the increase of anaerobic metabolism to compensate for the insufficient O_2 concentrations in the environment (van Hesswijk et al. 2005). In AB fish, the effectiveness of biochemical adjustments depends on the efficiency of the O_2 uptake from the air for the maintenance of aerobic metabolism. Otherwise, it is necessary to increase anaerobic metabolism to meet the energy requirements during aquatic environmental hypoxia (MacCormack et al. 2006).

Analyzing the variables associated with each step of the O_2 transport cascade permits the identification of the limiting factors for total gas exchange. Therefore, the main goal of this study is to analyze indicative parameters in each step of water- and air-breathing respiration in the AB armored catfish, *Pterygoplichthys anisitsi* (Teleostei, Loricariidae) and evaluate the respiratory adjustments of this fish to survive under hypoxic environmental conditions. The AB behavior under normoxia, progressive, and acute hypoxia, the respiratory responses under progressive hypoxia without access to atmospheric air, the hematological responses, hemoglobin characteristics, and intermediate metabolites in normoxia, under acute hypoxia and air exposure were analyzed to understand how each steps could contribute to the O_2 transport to the tissues and maintain the metabolism. *P. anisitsi* represents a suitable experimental model for studying air respiration in fish. This species is an AB species that belongs to the siluriforms, a group of fish shown by Hochachka and Lutz (2001) to be highly hypoxia-tolerant and use the stomach as an accessory AB organ (Cruz 2007; Cruz et al. 2009). In polluted regions, such as the Rio Preto, a river that is characterized by low O_2 concentrations and poor water quality for the sustaining aquatic life (CETESB 2010), *P. anisitsi* is the only fish species encountered.

Materials and methods

Animals

Adult specimens of the armored catfish *P. anisitsi* [$n = 68$; body mass (M_B) = 0.084–0.600 kg, mean ± SD = 0.28 ± 0.21 kg; total length (L_T) = 24–39 cm, mean ± SD = 31.80 ± 7.59 cm] were obtained from the Aquaculture Center of the São Paulo State University (CAUNESP) in Jaboticabal, São Paulo state, Brazil. The fish were maintained in the laboratory in a 1,000-L aquarium with continuous water flow and aeration at 24 ± 1 °C and were fed with commercial fish food and *Lactuta sativa* leaves. Feeding was stopped 24 h prior to experiments.

Experimental protocols

To study the different steps of the O_2 transport cascade, four different protocols were applied:

Protocol 1: AB behavior in fish with free access to atmospheric air in normoxia and under progressive and acute hypoxia

The experiments were performed using twenty animals in three experimental series (normoxia, N; progressive hypoxia, PH; and acute hypoxia, AH);

depending on fish size, each set consisted of two groups of three to four specimens in total that were placed in an aquarium (120 × 30 × 40 cm) with free access to atmospheric air. The specimens were placed into the aquarium under normoxic conditions (water oxygen partial pressure, PwO_2 = 130–140 mmHg) at 24 ± 1 °C, 18 h before the beginning of the experiments. The first experimental series measured was the AB behavior of the fish under normoxic conditions (PwO_2 = 130–140 mmHg), and the second experimental series measured was the AB behavior of the fish under acute hypoxia (PwO_2 = 20 mmHg). The reduction in the PwO_2 from normoxia to acute hypoxia, which took 30 min, was achieved by bubbling N_2 into the water and it was monitored continuously using a FAC-204A O_2 Analyzer (FAC, São Carlos, Brazil). Fish activity was recorded twice a day (at 11:00 to 14:00 h and at 18:00 to 21:00 h) using a video camera (JVC Video movie GR-AXM77UM). The first recording (11:00 to 14:00 h) occurred under natural light conditions, and the second recording (18:00 to 21:00 h) started late in the afternoon with nightfall occurring between 18:00 and 19:00 h. The third experimental series measured was the AB behavior of the fish under continuous progressive hypoxia (PwO_2 = 100, 70, 50, 40, 30, and 20 mmHg) over the course of 6 h during day time. Progressive hypoxia was achieved by bubbling N2 into the water, which was monitored continuously using a FAC-204A O_2 Analyzer. The AB behavior of fish, the AB frequency (breaths per hour), and the O_2 levels at the onset of air breathing during progressive hypoxia were evaluated in post-experimental analysis of the video footage.

Protocol 2: Respiratory responses to progressive hypoxia without access to atmospheric air

The respiratory responses to graded hypoxia without access to atmospheric air were assessed by measuring the routine O_2 consumption ($\dot{V}O_2$ = $mLO_2\ kg^{-1}\ h^{-1}$), gill ventilation ($\dot{V}_G$ = $mLH_2O\ kg^{-1}\ min^{-1}$), respiratory frequency (f_R = breaths min^{-1}), tidal volume (V_T = $mLH_2O\ kg^{-1}\ breath^{-1}$), and O_2 extraction from the ventilatory current (EO_2 = %) using the flow-through respirometry system (Fernandes and Rantin 1989). Briefly, fish (n = 6) were anesthetized using 0.01 % Benzocaine® solution (Synth), and the gills were artificially ventilated with 0.005 % Benzocaine® solution during the insertion of polyethylene catheters into the buccal and opercular cavities, which permitted the collection of water samples from each cavity and the measurement of the inspired (PiO_2) and expired (PeO_2) water PO_2, respectively. Thereafter, the fish were placed in the flow-through respirometer, which was placed inside a test chamber and left overnight to allow the fish to recover; the water flow through the respirometer was adjusted according to the size of the fish, so that the difference between inlet ($P_{in}O_2$) and outlet ($P_{out}O_2$) water from the respirometer was never higher than 10 %.

The PwO_2 was reduced gradually to different levels (PwO_2 = 100, 70, 50, 40, 30, and 20 mmHg) by bubbling N_2 into the water. Before measurements were taken at each PwO_2 level, the PwO_2 was maintained for 45 min to replace the water inside the respirometry system and to achieve PwO_2 stability inside the respirometer. The $P_{in}O_2$ and $P_{out}O_2$, as well as the PiO_2 and PeO_2, were measured by siphoning water via polyethylene catheters through thermostatted cuvettes housing O_2 electrodes connected to O_2 analyzers (FAC-204A).

$\dot{V}O_2$ was calculated as $\dot{V}O_2 = V_R(P_{in}O_2 - P_{out}O_2)\alpha O_2/M_B$,

where αO_2 is the solubility coefficient for O_2 in water and M_B is the body mass.

$\dot{V}_G$ was calculated as $\dot{V}_G = (P_{in}O_2 - P_{out}O_2)/(P_iO_2 - P_eO_2)V_R/M_B$

EO_2 was calculated as $EO_2 = 100(P_iO_2 - P_eO_2)P_iO_2$

and the f_R was recorded by connecting the buccal catheter to a Narco P-1000B pressure transducer that was coupled to the universal coupler of a Narco Narcotrace 40 physiograph. V_T was calculated by dividing the $\dot{V}_G$ by the f_R.

Protocol 3: Hemoglobinic measurements in normoxia and progressive hypoxia without access to the surface or exposure to the air

The fish were divided into three groups: normoxia (N, n = 6), progressive hypoxia (PH, n = 6), and air exposure (AE, n = 6). Normoxic fish were transferred randomly from the acclimation aquarium into an experimental opaque PVC box (150 L) that was divided into six compartments with one fish in each

compartment and kept in normoxic water for 24 h. Thereafter, blood was collected from the caudal vein of the fish using heparinized syringes.

Progressive hypoxic fish was exposed to hypoxia using the same flow-through respirometry system as described in protocol 2 but without the surgical procedures. The PwO2 was reduced gradually to 100, 70, 50, 40, 30, and 20 mmHg, and each PwO_2 level was maintained for 45 min before reduction to the next level until the PwO_2 reached 20 mmHg. After 45 min exposure to $PwO_2 = 20$ mmHg, the fish were removed from the respirometer and the blood was immediately collected from the caudal vein using heparinized syringes.

Air exposure fish were exposed to atmospheric air. The fish were transferred randomly from the acclimation aquarium into an experimental opaque PVC box (150 L) that was divided into six compartments with one fish in each compartment. The experimental box contained a thin layer of water to maintain the environmental humidity. After 24 h of air exposure, blood was collected from the caudal vein using heparinized syringes.

The hemoglobin (Hb) analysis was performed on whole blood and on stripped Hb. The oxygen equilibrium of the whole blood was calculated using the gasometric method. A suspension of unfractionated blood and yeast in an isotonic buffered solution (0.1 M bis–Tris/HCl and 0.1 M Tris/HCl 0.1 M) was placed in a YSI Oxygen Monitor (model 53), and the O_2 consumption was plotted using a recorder. Oxygen dissociation was determined as described by Johansen et al. (1978) and the P50 and Hill coefficient were calculated. The P_{50} values were converted into the logarithmic scale and were plotted against the final pH. The Bohr effect (ΔlogP50/ΔpH) was calculated from the O_2 dissociation curves obtained at different pH values.

To prepare the Hb hemolysate, the red blood cells were washed three times with 0.9 % NaCl, centrifuged and 15 mM Tris–EDTA (pH 8.0) was added into the packed erythrocytes at a ratio of 2:1 and the blood cells were subjected to three freeze-thawing treatments. Thereafter, the samples were centrifuged at 20,200 g (20 min) at 4 °C to free the hemolysate from the cellular debris. The supernatant Hb solution was then frozen at −20 °C until the O_2 equilibrium was measured and the electrophoresis analysis was performed.

The O_2 equilibrium of stripped Hb was determined at 20 °C using the spectrophotometric method described by Giardina and Amiconi (1981). The Hb supernatant was stripped of salt and organic phosphates by passing it through a 2.5 × 30 cm column of Sephadex G-25 resin equilibrated in 0.1 mM Tris–HCl, pH 8.0, with dithionite solution ($Na_2S_2O_4$). Hemoglobin and methemoglobin concentrations were estimated using the extinction coefficients for human Hb (Benesch et al. 1973). Samples containing more than 5 % methemoglobin (final concentration) were discarded.

The Hb pattern of *P. anisitsi* was determined using a horizontal electrophoresis system. The electrophoresis of individual hemolysates was performed using starch gels as described by Smithies (1955, 1959) and modified by Val et al. (1981). The starch gel was prepared using Tris–borate-EDTA buffer, pH 8.6, and 0.35 M borate buffer, pH 8.6. Hb migration was carried out at 4 °C, applying 5 V/cm and 1.25 mA/cm for 6 h. After Hb migration, the gel was stained with black starch to reveal the total proteins and was stained with benzidine solution to reveal the peroxidase activity of the Hb.

Protocol 4: Hematological and biochemical measurements in normoxia, acute hypoxia, and air exposure

The specimens were divided into the following 3 groups: normoxia (N, $n = 6$), the fish were maintained in normoxic water ($PwO_2 = 140$ mmHg) for 24 h; acute hypoxia (AH, $n = 24$), the fish were submitted to acute hypoxia ($PwO_2 = 20$ mmHg) without access to atmospheric air for 1, 2, 3, and 4 h ($n = 6$ in each experimental period); and air exposure (AE, $n = 6$), the fish were exposed to atmospheric air for 24 h. The fish from each group were transferred randomly from the acclimation aquarium (1,000 L) into an experimental opaque PVC box (150 L) that was divided into six compartments with one fish in each compartment. Fish from groups N and AH in each compartment shared the same water. Air exposure fish (AE) were placed individually in compartments containing a thin layer of water that was kept in the experimental box to conserve humidity and to prevent dehydration of the fish. After each experimental period, blood was obtained through the puncture of the caudal vein using heparinized syringes and hematological analyses and plasma glucose, lactate and pyruvate measurements were taken. Thereafter, the fish were anesthetized

(Benzocaine®, Synth) and killed to obtain samples of liver and white muscle for glucose, lactate, pyruvate, and glycogen analyses.

The blood pH, hematocrit (Ht, two readings using a percentage table after centrifugation), hemoglobin (Hb, measured using the cyanomethemoglobin method with Drabkin solution and read at an absorbance of 420 nm), and blood cell count (RBC, determined using a Neubauer counting-camera) were determined for each blood sample. The derived hematological parameters, such as the mean cell hemoglobin concentration (MCHC), the mean cell volume (MCV), and the mean cell hemoglobin (MCH), were calculated using the Ht, [Hb], and RBC data. The remaining blood was centrifuged at 12,000 g, and the plasma was separated for biochemical analyses.

The intermediate metabolites were measured in the plasma and in the liver and white muscle homogenates. Trichloroacetic acid (TCA, 20 %) was added to the plasma, which was centrifuged at 12,000 g (3 min). The liver and white muscle tissue were mechanically homogenized in 20 % TCA and centrifuged at 12,000 g (3 min). The plasma and tissue supernatants were used as acid extracts for glucose, lactate, and pyruvate measurements. All measurements were taken using a spectrophotometer Biochron Libra S32 (Biochrom, UK). Plasma glucose was measured using the glucose oxidase reaction at 525 nm (Trinder 1969), and liver and muscle glucose were determined using phenol–sulfuric acid at 480 nm (Dubois et al. 1956). Lactate was determined using p-hydroxyphenyl phenol at 570 nm (Harrower and Brown 1972), and pyruvate was determined using dinitrophenyl hydrazine-NaOH at 440 nm (Lu 1939). Glycogen in the liver and white muscle samples was measured following the dissolving of the tissues in 1 mL of KOH 6.0 N in a boiling water bath (3 min). The liver and white muscle alkaline extract were used to measure glycogen after alcohol precipitation using the method described by Bidinotto et al. (1997). The reducing sugar content was measured using the method described by Dubois et al. (1956) at 480 nm and was expressed as μM of glucosil-glucose g^{-1} tissue.

Statistics

The respiratory variables are presented as the mean and the range, whereas the hematological and metabolic data are presented as the mean ± standard deviation. The data normality was verified using the D'Agostino-Pearson test. The respiratory variables ranges were compared using the Kruskal–Wallis nonparametric test followed by the Dunn test for multiple comparisons ($p < 0.05$; Zar 1999). Hematological and metabolic data were compared using a one-way ANOVA followed by Tukey's test for multiple comparisons ($p < 0.05$). The functional behavior of Hb was presented as the amplitude of the Bohr effect obtained by linear regression analyses using the Microcal Origin 6.0 software.

Results

AB behavior in normoxia and under progressive and acute hypoxia with free access to atmospheric air

In normoxia, during daylight hours, the *P. anisitsi* did not breathe atmospheric air. The fish remained on the aquarium bottom, alternating between long periods of inactivity and brief periods of slow movements. In the late afternoon, the fish became more active and ascended to the water surface to gulp air (approximately 2 AB h^{-1}) and quickly returned to the bottom of the aquarium. In total darkness, the frequency of AB increased (AB = 5 ± 1 h^{-1}), with the fish swimming vertically in the water column and holding their mouths open above the water surface for few minutes before returning to the aquarium bottom. No AB behavior was observed during the daytime progressive hypoxia, even at PwO_2 = 20 mmHg. Under these conditions, most fish exhibited apnea that can be described as a sequence of the following events: (1) closing opercula, (2) lip protrusion, (3) pulsating movements of the mouth for nearly 20 s forcing the water to enter and to leave exclusively through the mouth, and (4) complete closure of the mouth. When fish were submitted to acute hypoxic conditions (PwO_2 = 20 mmHg), during the day, 70 % of the fish exhibited AB behavior at a frequency of 4 ± 1 AB h^{-1}. No measurements were taken during night.

Respiratory responses to progressive hypoxia without access to atmospheric air

The $\dot{V}O_2$ and the gill respiratory parameters ($\dot{V}_G$, V_T, EO_2, f_R) of *P. anisitsi* under different PwO_2 without

Table 1 Mean oxygen consumption ($\dot{V}O_2$) and the ventilatory parameter, gill ventilation ($\dot{V}_G$), respiratory frequency (f_R), tidal volume (V_T), and O_2 extraction from the ventilatory current (EO_2) values during reductions of O_2 tensions in water (PwO_2) and inspired water (PiO_2) of *P. anisitsi* submitted to graded hypoxia without access to the surface

PwO_2 (mmHg)	PiO_2 (mmHg)	$\dot{V}O_2$ ($mLO_2\ kg^{-1}\ h^{-1}$)	$\dot{V}_G$ ($mLH_2O\ kg^{-1}\ min^{-1}$)	V_T ($mLO_2\ kg^{-1}$ $breath^{-1}$)	EO_2 (%)	f_R ($breath\ min^{-1}$)	*n*
140	132 (136–121)	52 (30–97)[a]	734 (603–1452)[a]	6 (5–11)[a]	20 (12–38)[a]	117 (106–132)[a]	6
100	93 (101–86)	42 (24–56)[a]	501 (253–1843)[a]	5 (2–15)[a]	27 (12–66)[a]	122 (118–144)[a]	6
70	68 (74–58)	39 (27–46)[a]	936 (413–8710)[a]	7 (3–66)[a]	26 (2–59)[a]	127 (120–142)[a]	4
50	49 (54–41)	36 (30–58)[a]	1496 (474–11982)[a]	10 (4–107)[a]	25 (3–56)[a]	118 (110–124)[a]	4
40	40 (42–38)	12 (9–32)[b]	1290 (513–6490)[a]	9 (2–50)[a]	14 (2–62)[a]	132 (122–218)[a]	4
30	28	34	9890	159	5	62	1
20	13	5	23076	262	1	88	1

The maximum and minimum values are given in parentheses. Statistical differences among treatments are indicated by dissimilar letters. The $\dot{V}O_2$ and respiratory parameter values of $n = 1$ were not considered for statistical analyses

access to atmospheric air are shown in Table 1. The differences between the PwO_2 and PiO_2 are due to the inspired and expired water mixing inside the respirometer and the water reflux from the opercular cavities into the buccal cavity during the end phase of the respiratory ventilation cycle.

The $\dot{V}O_2$ in normoxia was low ($\dot{V}O_2 = 52\ mLO_2\ kg^{-1}\ h^{-1}$), varying between 30 to 97 $mLO_2\ kg^{-1}\ h^{-1}$, and was kept approximately constant until it reached a $PwO_2 = 50$ mmHg ($PiO_2 = 49$ mmHg). At $PwO_2 = 40$ mmHg ($PiO_2 = 40$ mmHg), the $\dot{V}O_2$ decreased significantly in relation to normoxia ($p < 0.05$). The critical oxygen tension (PcO_2) was calculated as $PiO_2 = 47 \pm 2$ mmHg. From the six specimens submitted to progressive hypoxia without access to atmospheric air, 2 fish stopped ventilation at a PwO_2 lower than 70 mmHg and 2 fish stopped ventilation at a PiO2 lower than 40 mmHg after which the experiment was ended. At O_2 tensions lower than $PwO_2 = 40$ mmHg, the fish presented continuous apnea behavior and the respiratory measurements were then suspended. Only one fish maintained respiration at a PwO_2 lower than 40 mmHg in water.

The gill ventilation ($\dot{V}_G$) and respiratory frequency (f_R) exhibited a tendency to increase during progressive hypoxia but did not differ significantly from normoxia ($p > 0.05$ and $p > 0.05$, respectively) (Table 1). The fish that presented apnea behavior at a very low PwO_2 exhibited a continuous pattern of respiratory frequency, whereas the fish that maintained ventilation exhibited a burst of respiration followed by short periods of apnea (2.6 ± 0.7 s). The V_T was kept relatively constant under progressive hypoxia and the EO_2, which was very low in normoxia, did not change under hypoxia (Table 1).

Hemoglobinic parameters in normoxia, progressive hypoxia without access to the surface, and air exposure

The Hb hemolysates of all *P. anisitsi* analyzed showed ten hemoglobinic components that were numbered according to decreasing anodic mobility; two groups of rapid anodic components (I, II, III and IV, V, VI), a group of anodic components with intermediate migration (VII and VIII), one anodic component with slow migration (IX), and one cathodic component (X).

Figure 1 shows the whole blood Hb–O_2 and stripped Hb–O_2 affinities (log P50) and the cooperativity (n_{Hill}) in relation to pH for *P. anisitsi*. Table 2 shows the values of the Bohr (Φ) and Hill (n_{Hill}) coefficients along with pH, the P_{50}, and the log P_{50}. The slopes of the Bohr coefficient of whole blood from the fish kept under hypoxic conditions ($\Phi = -0.24$) was steeper than those from the fish exposed to atmospheric air ($\Phi = -0.10$) and those obtained for stripped Hb. No Bohr effect ($\Phi = -0.02$) was observed in fish kept in normoxic conditions. Under the three tested conditions (normoxia, hypoxia, and air exposure), the P_{50} values of the whole blood were greater than the stripped Hb ($p < 0.05$), and the P_{50} values of fish exposed to hypoxia were lower than those kept in normoxia or exposed to atmospheric air.

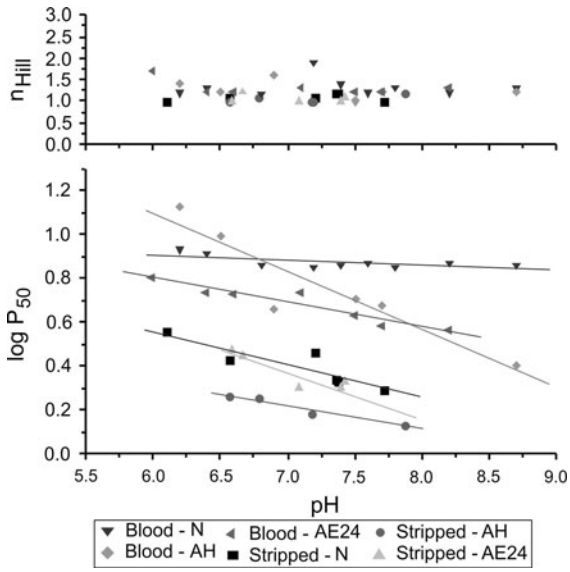

Fig. 1 Hemoglobin–O2 affinity (log P_{50}) and cooperativity (n_{Hill}) of whole blood and stripped hemoglobin in *P. anisitsi* under normoxia (N), acute hypoxia (AH), and 24-h air exposure (AE24). The correlation coefficient (r^2) of regressions varied from 0.89 to 0.98

The Hill coefficients of the whole blood of fish kept in normoxia ($n_{Hill} = 1.7$) and hypoxia ($n_{Hill} = 1.6$) showed high cooperativity, whereas the values of whole blood after air exposure ($n_{Hill} = 1.3$) and of stripped Hb ($n_{Hill} = 1.0$–1.1) in all study conditions showed low cooperativity values.

Hematological parameters in normoxia, acute hypoxia without access to atmospheric air, and air exposure

In normoxia, the Ht, Hb concentration, and RBC were 23 ± 4.12 %, 6.71 ± 1.21 g dL^{-1}, and $1.05\ 10^6$ mm^{-1}, respectively, and the blood pH was 7.81 ± 0.06 (Fig. 2a, b, c, g). During acute hypoxia, the Ht increased significantly after 1 h ($p < 0.001$), 2 h ($p < 0.01$), and 3 h ($p < 0.05$) exposure (Fig. 2a), the Hb concentration increased after 1-h ($p < 0.01$) exposure (Fig. 2b), and the RBC increased significantly after 1 h and 2 h ($p < 0.05$; Fig. 2c) compared to the normoxic group. The MCHC decreased after 2 h ($p < 0.01$) (Fig. 2d) and 3 h ($p < 0.05$) of hypoxia exposure, whereas the MCH (Fig. 1e) and MVC (Fig. 2f) remained unaltered. The pH remained unaltered during the first 2 h of exposure to hypoxia and decreased significantly after 3 h and 4 h ($p < 0.01$) exposure (Fig. 2g). No significant differences occurred in the hematological parameters after 24 h of air exposure.

Intermediate metabolites in normoxia, acute hypoxia without access to atmospheric air, and air exposure

In the plasma, the glucose levels increased (Fig. 3a) during hypoxia compared to the normoxic group, the lactate levels increased after 3 h ($p < 0.01$) and 4 h ($p < 0.001$) hypoxia (Fig. 3b), and the pyruvate concentrations increased after 1 h hypoxia ($p < 0.05$; Fig. 3c). After 24-h air exposure, the glucose and pyruvate levels increased ($p < 0.05$) and the lactate levels did not change (Fig. 3a, b, c).

The hepatic glucose concentrations remained unaltered over the first 2 h of hypoxia and decreased significantly after 3 and 4 h ($p < 0.01$; Fig. 4a), the lactate levels increased after 4 h ($p < 0.01$) hypoxia (Fig. 4b), and the pyruvate concentrations decreased significantly after 4 h hypoxia ($p < 0.001$; Fig. 4c).

Table 2 Bohr coefficient (ϕ), P_{50}, log P_{50}, and cooperativity (n) of whole blood and stripped hemoglobin from *P. anisitsi* in normoxia, acute hypoxia, and 24-h air exposure

	Bohr (Φ)	P_{50}	Log P_{50}	Hill (n_{Hill})
Blood normoxia	−0.02	7.02 ± 0.21^a	0.85 ± 0.01^a	1.70
Blood hypoxia	−0.24	4.59 ± 0.18^b	0.66 ± 0.02^b	1.60
Blood air 24 h	−0.10	5.56 ± 0.46^a	0.74 ± 0.03^a	1.30
Stripped normoxia	−0.14	2.93 ± 0.16^c	0.46 ± 0.02^c	1.10
Stripped hypoxia	−0.10	1.53 ± 0.23^d	0.18 ± 0.07^d	1.00
Stripped air 24 h	−0.15	2.00 ± 0.14^c	0.30 ± 0.03^c	1.00

The correlation coefficient (r^2) was 0.95 for regressions. P_{50} and log P_{50} values are mean ± SD; statistical differences among treatments are indicated by dissimilar letters

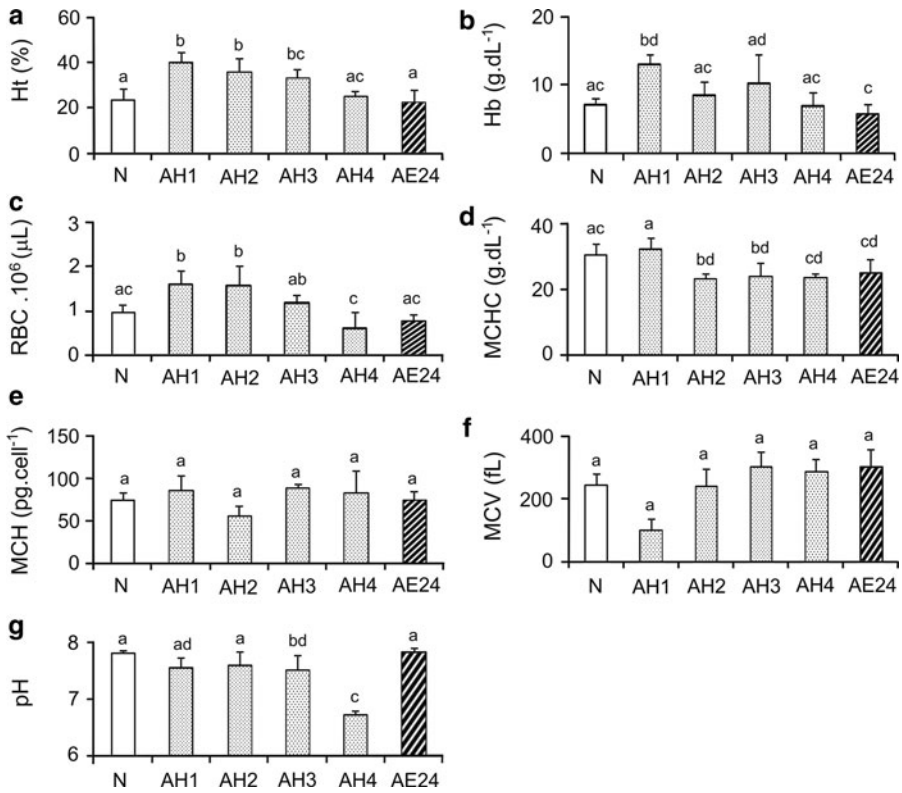

Fig. 2 Hematological parameters of *P. anisitsi*. **a** hematocrit (Ht), **b** hemoglobin concentration (Hb), **c** red blood cell (RBC) and **d** mean cell hemoglobin concentration (MCHC), **e** mean cell hemoglobin (MCH), **f** mean cell volume (MCV), and **g** pH, in normoxia (N), acute hypoxia (AH) after 1 h (AH1), 2 h (AH2), 3 h (AH3), 4 h (AH4), and after 24 h of air exposure (AE24). Mean ± SD. Statistical differences among treatments are indicated by *dissimilar letters*

The hepatic glycogen levels dropped significantly after 3 h ($p < 0.05$) and 4 h ($p < 0.001$) hypoxia (Fig. 4d). After 24 h of air exposure, the hepatic glucose levels decreased ($p < 0.01$; Fig. 4a), the lactate and pyruvate concentrations (Fig. 4b, c) and the glycogen content (Fig. 4d) did not change.

In white muscles, the glucose concentrations decreased after 4 h ($p < 0.01$) of hypoxia and after 24 h of air exposure ($p < 0.01$; Fig. 5a); the lactate levels increased ($p < 0.05$) after 4 h of hypoxia (Fig. 5b); and the pyruvate concentrations increased significantly only after 24 h of air exposure ($p < 0.001$; Fig. 5c). No significant differences were found in the glycogen contents in the white muscles under any experimental conditions (Fig. 5d).

Discussion

This study demonstrates that the AB armored catfish *P. anisitsi* depends on the adjustments of respiratory process to maintain the metabolic demands when faced with varying environmental conditions.

AB behavior in fish with free access to atmospheric air in normoxia and under progressive and acute hypoxia

In accordance with the classification of AB fish types proposed by Graham (1997), *P. anisitsi* is considered a continuous, but non-obligatory, AB fish. This classification was based on the AB behavior of the fish and the environmental factors that affect fish respiration. In normoxic water, continuous non-obligatory air breathers, such as *P. anisitsi*, do not require atmospheric air to survive (Graham 1997). In general, most facultative (non-obligatory) AB loricariid fish are not continuous air breathers but begin to breathe air when the PwO_2 drops to the threshold level, which triggers this behavior (Mattias et al. 1998; Takasusuki et al. 1998). This hypoxic threshold usually coincides with the critical oxygen pressure (PcO_2) and/or the appearance of anaerobic end products in the tissues of the fish (Chapman and McKenzie 2009).

Air gulping during the period of lower luminosity and darkness, under normoxia, may be related to the daily cyclical activity pattern of *P. anisitsi*. Cruz and Langeani (2000) reported higher swimming activity of

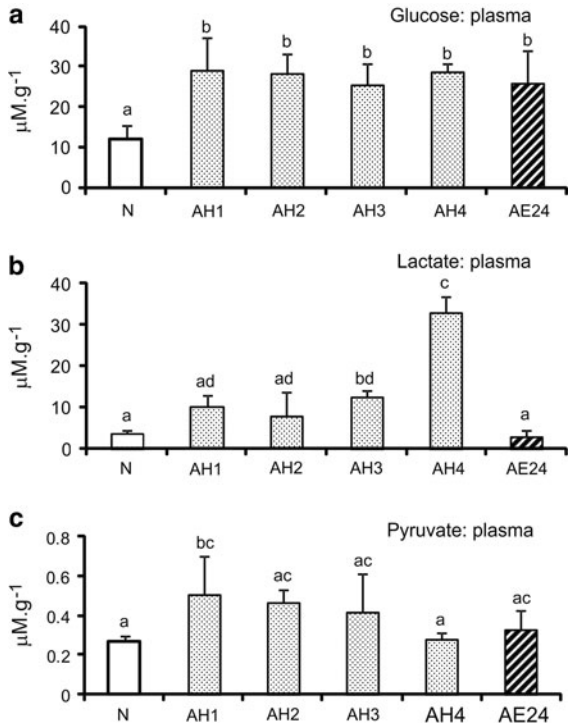

Fig. 3 Plasma **a** glucose, **b** lactate, and **c** pyruvate concentrations of *P. anisitsi*, in normoxia (N), acute hypoxia (AH) after 1 h (AH1), 2 h (AH2), 3 h (AH3), 4 h (AH4), and after 24 h of air exposure (AE24). Mean ± standard deviation. Statistical differences among treatments are indicated by *dissimilar letters*

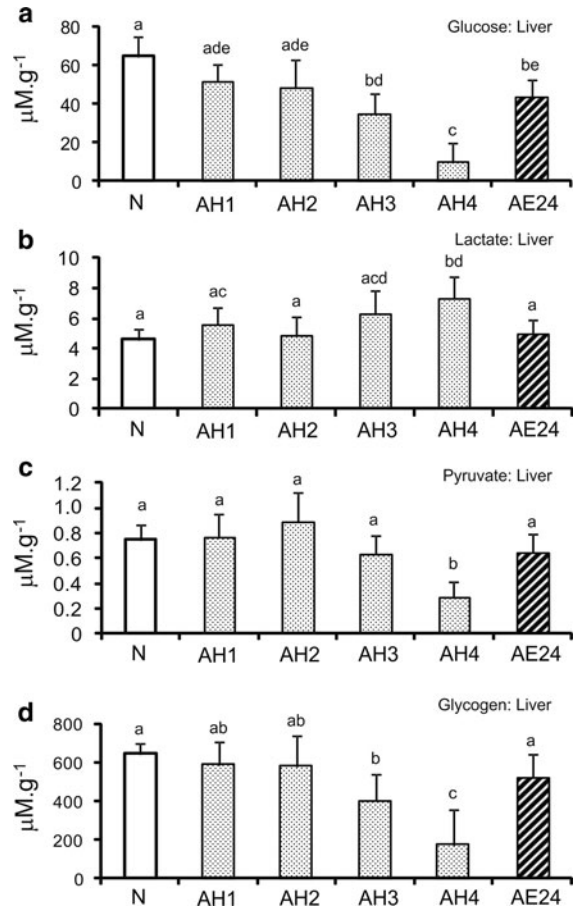

Fig. 4 Liver **a** glucose, **b** lactate, **c** pyruvate, and **d** glycogen concentrations of *P. anisitsi*, in normoxia (N), acute hypoxia (AH) after 1 h (AH1), 2 h (AH2), 3 h (AH3), 4 h (AH4), and after 24 h of air exposure (AE24). Mean ± standard deviation. Statistical differences among treatments are indicated by *dissimilar letters*

P. anisitsi at night than during the day. Similar AB behavior was described for *Glyptoperichthyes gibbeceps*, another AB loricariid species that remains on the river bottom during the middle of the day and explores the upper water column more frequently at night and in the early morning (MacCormack et al. 2003). Nocturnal air gulping is considered an anti-predatory behavior for other AB fish because the activity of fish-eating birds is greatly reduced at night (Gee and Graham 1978, Gee 1980). However, some species, as *Clarias gariepinus*, exhibit continuous air breathing during the day (2 AB h^{-1}) under normoxia and increased air respiration during progressive hypoxia, reaching up to 11 ± 2 AB h^{-1} in severe hypoxia (PwO2 = 10 mmHg) (Belão 2010).

The reduction of routine spontaneous activity in hypoxia during the day saves energy expenditure, and the intermittent ventilation (apnea periods) exhibited by *P. anisitsi* may be an adaptive strategy to minimize the cost of ventilation (Milsson 1991).

Respiratory responses to progressive hypoxia without access to atmospheric air (aquatic respiration)

The low routine $\dot{V}O_2$ of *P. anisitsi* under normoxic and moderate hypoxic conditions varying from 30 to 97 $mLO_2\ kg^{-1}\ h^{-1}$ were within the range of other loricariid species, such as *Ancistrus chagresi* (Graham 1983), *Hypostomus plecostomus* (Perna and Fernandes 1996), *Rhinelepis strigosa* (Takasusuki et al. 1998), and *Hypostomus regani* (Mattias et al. 1998; Nelson et al. 2007). It is thought that the low $\dot{V}O_2$ is due to the large amount of tissues with a low metabolic rate, such as bone plates, in these fish (Takasusuki et al. 1998). However, the larger variation in $\dot{V}O_2$ and in most

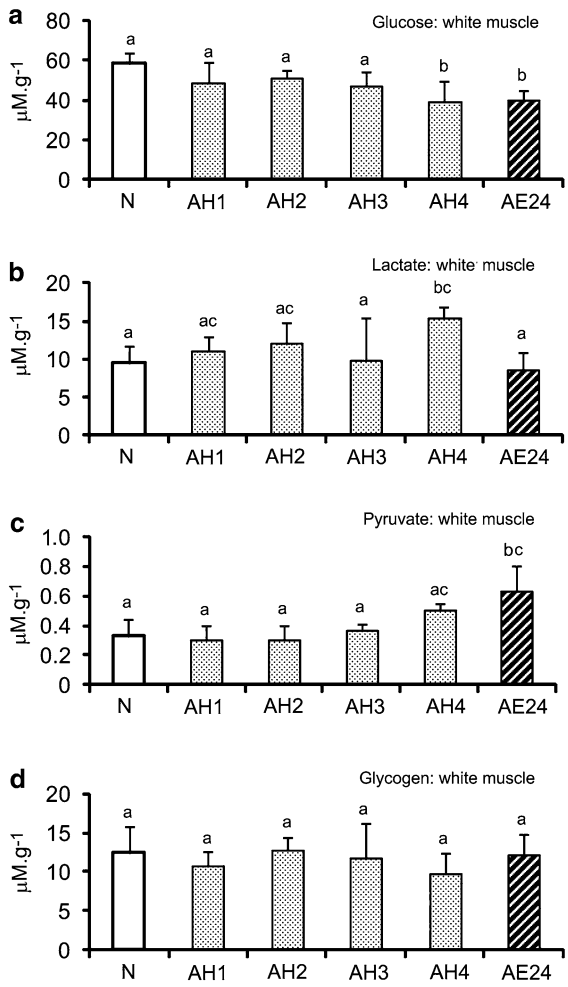

Fig. 5 White muscle **a** glucose, **b** lactate, **c** pyruvate, and **d** glycogen concentrations of *P. anisitsi*, in normoxia (N), acute hypoxia (AH) after 1 h (AH1), 2 h (AH2), 3 h (AH3), 4 h (AH4), and after 24 h of air exposure (AE24). Mean ± standard deviation. Statistical differences among treatments are indicated by *dissimilar letters*

ventilatory parameters in *P. anisitsi* compared to other loricariid species (Mattias et al. 1998; Takasusuki et al. 1998) may be due to different levels of individual stress tolerance to experimental procedures (cannulation, foreign tank upon arousal from anesthesia, etc.), as has been suggested by Nelson et al. (2007). During progressive hypoxia without access to atmospheric air, *P. anisitsi* were shown to regulate oxygen from normoxia (PwO_2 = 140 mmHg, PiO_2 = 132 mmHg) to moderate hypoxia (PwO_2 = 50 mmHg, PiO_2 = 49 mmHg). The calculated PcO_2 (PiO_2 = 47 ± 2 mmHg) was higher than in other loricariid species, such as *H. regani* (PiO_2 = 34 mmHg; Mattias et al. 1998; Nelson et al. 2007) and *R. strigosa* (PiO_2 = 24 mmHg; Takasusuki et al. 1998), but similar to other AB fish (Hughes and Singh 1971; Oliveira et al. 2004; Belão 2010).

In fish, the increase in the $\dot{V}_G$ is produced by increasing the f_R and V_T to compensate for the PwO_2 reduction. Increases in the V_T are advantageous because this incurs lower energy costs for ventilation, whereas increasing the f_R incurs a higher metabolic cost for respiration and is limited by internal muscle viscosity and high water viscosity (Rantin et al. 1992; Fernandes and Rantin 1994). Hyperventilation of the gills by increasing the V_T instead of the f_R was described for the loricariids *H. regani* (Mattias et al. 1998; Fernandes et al. 1999; Nelson et al. 2007) and *Rhinelepis strigosa* (Takasusuki et al. 1998) as well as for other air-breathing and water-breathing species (Mattias et al. 1996; Souza et al. 2001; Oliveira et al. 2004). However, *P. anisitsi* exhibited some inability to perform ventilatory adjustments during progressive hypoxia to sustain its oxygen requirements. A similar reduced ability to increase ventilation was found in other AB fish (Graham et al. 1987; Graham and Wegner 2010). The intermittent respiration pattern as well the apnea that was observed with and without access to atmospheric air in this species of fish reinforces the hypothesis that this respiratory behavior may be a response that reduces the cost of gill ventilation. The AB fish, *Synbranchus marmoratus*, exhibits very low breath frequency (27.9 ± 3.1 ventilation min^{-1}) in normoxia and does not ventilate its gills; therefore, it continuously exhibits regular periods of apnea, including during graded hypoxia until the PwO_2 is as low as 5–15 mmHg (Graham and Baird 1984). When the air breathing is initiated at PwO_2 ~30 mmHg, the fish usually stop aquatic ventilation, which minimizes the potential for transbranchial O_2 loss (Bicudo and Johansen 1979; Graham and Wegner 2010).

Hematology and the hemoglobin properties

The changes in the hematological parameters during the first 2 h under conditions of severe hypoxia is a general response to improve the O_2-carrying capacity of the blood (Nikinmaa 2002), suggesting blood cell release by spleen contraction. However, these responses may increase the energy expenditure due to

a greater cardiovascular effort and the breakdown of erythrocyte production (Caldwell and Hinshaw 1994). The return of the hematological parameters in the 3 and 4 h following hypoxia exposure suggests the transition to other mechanisms to maintain oxygen delivery.

The Hbs in *P. anisitsi* can be classified as class II Hbs, according to Hochachka and Somero (2002), which include species expressing anodic Hbs with a normal Bohr effect, cathodic Hbs with a high affinity for O_2, and a small or even reverse Bohr effect in which the O_2 affinity is not affected by pH. From the ten hemoglobinic components found in the blood, nine are anodic and exhibit different migration rates and one is cathodic. Multiple Hb components are an important advantage for animals living in a highly variable environment that includes daily and seasonal changes (Tamburrini et al. 2001). The presence of a multiple Hb components enables *P. anisitsi* to inhabit hypoxic and anoxic environments, such as a number of regions along the Preto River, SP, Brazil, where it is the only species consistently found (CETESB 2010). The cathodic Hb component contributes to maintaining cellular oxygenation under very low oxygen conditions (Fago et al. 1995; Tamburrini et al. 2001). The lower P_{50} values of whole blood and stripped Hb of fish kept under hypoxia indicate a higher Hb–O_2 affinity compared with fish kept in normoxia or exposed to atmospheric air.

The increased whole blood Bohr effect under hypoxic conditions is beneficial to fish (Jensen 2004) because it increases the efficiency of O_2 uptake in the gills and O_2 release in the tissues. The increase in Hb–O_2 affinity during periods of aquatic O_2 restriction without access to atmospheric air together with the large respiratory surface area that is similar in water-breathing species (Cruz 2007) represents an advantage for *P. anisitsi* to maintain the metabolic processes during short periods of hypoxia. However, the reduction in plasma pH levels after 4 h of hypoxia indicates that anaerobic metabolism (increased lactate concentration, Fig. 3b) are initiated after long periods of hypoxia (Hochachka and Somero 2002). The low plasma pH also indicates an inefficiency for compensating for the increase in acidic metabolite derivates.

Conversely, the similarity between the hematological parameters and the Hb functional characteristics in the fish after air exposure and in normoxic water suggests a high adaptation of this species to support air exposure. The large respiratory surface of the stomach of *P. anisitsi* (Cruz et al. 2009) appears to be sufficient for oxygen uptake and for the maintenance of aerobic scope. However, further studies are needed to determine how CO_2 is excreted under these conditions because no change in the blood pH was observed after 24 h of exposure to the air.

Intermediate metabolism

In general, hypoxia-tolerant fish species present high liver glycogen contents (Hochachka and Somero 2002; Chippari-Gomes et al. 2005) and *P. anisitsi,* in normoxia, exhibits higher hepatic glycogen contents compared with other hypoxia-tolerant fish, such as *Pterygoplichthys pardalis* (MacCormack et al. 2006), *H. regani* (Bidinotto et al. 1997), and *Hoplias malabaricus* (Moraes et al. 1996). Under hypoxia, many vertebrates use anaerobic metabolism (Hochachka and Somero 2002) to survive temporarily. An anaerobic response was identified in *P. anisitsi* exposed to hypoxic conditions without access to atmospheric air. The plasma glucose level increased concomitant with the decrease in hepatic glycogen and glucose content suggesting that glycogen is mobilized and may be the primary fuel used to maintain anaerobic metabolism during acute hypoxia. The reduction in glucose and the increase in lactate found in the white muscle indicate a lactic fermentative process in this tissue after 4 h of acute hypoxia and suggests similar response patterns to those found in *P. pardalis* (MacCormack et al. 2006) under hypoxic conditions and *S. marmoratus* after undergoing semi-estivation for 15 days (Moraes et al. 2005). The reduction in muscle glucose, together with the 25 % decrease in liver glycogen levels, the 40 % increase in muscle lactate levels, and the 800 % increase in blood lactate, suggests that anaerobic pathways are at their upper limits and the energy reserves of *P. anisitsi* are exhausted during the 4-h exposure to acute hypoxia without access to air. Fish exposed to air are able to obtain sufficient O_2 to support aerobic metabolism, and the hepatic glycogen stores are not used. However, the significant reduction in hepatic and muscle glucose, resulting in plasma hyperglycemia, may represent a stress response to air exposure. The increase in pyruvate together with unchanged muscle and plasma lactate levels suggests a possible increase in protein catabolism, as shown by Ip et al. (2001) for the amphibious fish *Boleophthalmus boddaerti*.

Conclusions

The air-breathing behavior and the physiological responses to hypoxia, air exposure, and the biochemical characteristics of Hbs allow *P. anisitsi* to maintain metabolic rate in different environmental conditions. *P. anisitsi* are continuous non-obligatory air-breathing fish that exhibit a high tolerance to hypoxia. Under normoxia and moderate hypoxia, these fish are oxygen regulators ($PcO2 = 49$ mmHg) and sustain O_2 uptake with non-significant changes in ventilation, probably due to the high Hb–O_2 affinity of their hemoglobin, mainly the cathodic component, and the large gill surface area (Cruz 2007), which is similar to that of water-breathing fish. Under severe hypoxia, *P. anisitsi* exhibited long periods of apnea and a high anaerobic capacity for up to 3–4 h of hypoxia without air breathing. These physiological responses under hypoxia favor this species during episodes of low O_2 tensions found in its natural habitats, including the absence and/or low air-breathing frequency during the day, which protects the fish from aerial predators. The similar physiological and biochemical traits under air exposure and normoxic conditions indicate the use of aerobic metabolism. It is likely that the functional respiratory surface of the stomach, as an AB organ (Cruz et al. 2009), during air exposure allows O_2 delivery to be maintained at sufficient levels to fulfill demand of the fish.

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