Journal of FISH BIOLOGY

fsbi An International Society for Fish Biology

Journal of Fish Biology (2016)
doi:10.1111/jfb.13009, available online at wileyonlinelibrary.com

In vitro effects of increased temperature and decreased pH on blood oxygen affinity of 10 fish species of the Amazon

A. L. Val*†, M. de N. Paula-Silva*, V. M. F. Almeida-Val* and C. M. Wood*‡

**Laboratory of Ecophysiology and Molecular Evolution, Brazilian National Institute for Research of the Amazon, Manaus, AM, Brazil and ‡Department of Zoology, University of British Columbia, Vancouver, BC, V6T 1Z4 Canada*

Blood-O_2 affinities (P_{50}) were measured over a physiologically relevant pH range at 31 (highest temperature average of Rio Negro over the last 8 years), 33 and 35° C for 10 species of the Rio Negro, aiming to test the acute effects of temperature foreseen by the IPCC (Intergovernmental Panel on Climate Change) for coming years. The animals were collected during an expedition to the Anavilhanas Islands of the Rio Negro, 110 km upstream from Manaus (2° 23′ 41″ S; 60° 55′ 14″ W). *Hoplias malabaricus* showed higher blood-O_2 sensitivity to pH changes (Bohr effect, $\Phi = \Delta \log_{10} P_{50}\, \Delta pH^{-1}$) at both 31° C ($\Phi = -0{\cdot}44$) and 35° C ($\Phi = -0{\cdot}26$) compared to *Osteoglossum bicirrhosum* ($\Phi = -0{\cdot}54$ at 31° C and $\Phi = -0{\cdot}58$ at 35° C), but lower P_{50} under most conditions, and a greater sensitivity of P_{50} to temperature. Two out of the 10 analysed species had significant increases of P_{50} (lower blood-O_2 affinity) at the highest temperature throughout the pH range tested. For all other species, a minor increase of P_{50} over the assay-tested temperatures was observed, although all presented a normal Bohr effect. Overall, a diversity of intensities of pH and temperature effects on blood-O_2 affinities was observed, which seems to be connected to the biological characteristics of the analysed species. Thermal disturbances in their habitats, likely to occur due to the global warming, would impair blood-O_2 binding and unloading in some of the analysed fish species. Copyright © 2016 John Wiley & Sons, Ltd.

Key words: blood-O_2 binding; Bohr effect; climate change; global warming; Rio Negro.

INTRODUCTION

In addition to its biological diversity, the Amazon is characterized by extreme environmental conditions, such as the acidic water of Rio Negro, the frequent hypoxic condition in many water bodies, high stable temperatures all year and high levels of CO_2 and H_2S during the low water season (Val & Almeida-Val, 1995). Many fish species of the Amazon have developed adaptive strategies at all levels of biological organization to face these environmental conditions (Fyhn *et al.*, 1979; Val & Almeida-Val, 1995; McCormack *et al.*, 2003; Wood *et al.*, 2009). The foreseen climate changes, however, will probably exacerbate these already extreme environmental conditions (Nobre, 2014), and may push the fishes of the Amazon to the limits of their adaptive abilities.

†Author to whom correspondence should be addressed: Tel.: +55 92 36833189; email: dalval@inpa.gov.br

Many species may be at risk under the global warming foreseen for the near future (Tewksbury *et al.*, 2008; Somero, 2010; Rummer *et al.*, 2014), including the many fish species living in the Rio Negro. An analysis of blood-O_2 affinities of fish species belonging to unrelated phylogenetic groups and having different habits may shed light on their ability to survive warmer and more acidic environmental scenarios. The fishes of the Amazon are indeed a key group for such analysis, as they include representatives from the Chondrichthyes, such as the freshwater stingrays, up to the most specialized ones, such as the cichlids (Nelson, 1994) and closely related species that have different lifestyles.

Owing to climate change, mean freshwater temperatures, including the water of the Amazon, are predicted to rise by up to 4° C (IPCC, 2013). In many Amazonian habitats, such as the shoreline of floodplain areas, the water temperature changes may be even higher as a consequence of other regional events (Sioli, 1984; Caraballo *et al.*, 2014). These situations, *i.e.* lower pH and higher temperatures, will pose significant new challenges to fishes to maintain oxygen supply to respiring tissues (Almeida-Val *et al.*, 2006; Holt & Jorgensen, 2015). The red blood cells contain haemoglobin that carries oxygen from the environment to respiring tissues. The red blood cell micro-environment must be adjusted according to both the environmental changes, such as increased temperature and lower dissolved oxygen, and the demand of respiring tissues, which muscles need during exercise, to secure oxygen transfer under adverse conditions. A diversity of haemoglobin (Hb) isoforms has been found in fishes and their concentrations are adaptively regulated in many species (Fyhn *et al.*, 1979; Val *et al.*, 1987; Weber *et al.*, 2000). The structural diversity, genetically encoded, determines the intrinsic O_2 binding properties of these Hb isoforms. The Hb-O_2 binding properties, however, are also determined by the micro-environment under which they operate (*i.e.* intra-erythrocytic chemistry), where elevations in protons (lowered pH), CO_2, chloride, organic phosphates and other anions modulate Hb-O_2 affinity favouring O_2 unloading to acidic oxygen-poor tissues, but making O_2 uptake at the respiratory surface more difficult (Weber & Campbell, 2010).

Increased temperature also causes a decreased Hb-O_2 affinity, which favours oxygen unloading to tissues, but which can be detrimental for Hb-O_2 binding at the gills. As water temperature increases, dissolved oxygen decreases and metabolic demand for oxygen increases. So, increased temperature would represent a significant constraint for fishes of the Amazon that already face low oxygen availability. Consequently, what are the effects of increased temperature on fishes of the Amazon belonging to different groups and having different habits?

The average of highest temperatures for Rio Negro waters over the last 8 years is 31° C (pers. data) and the climate scenarios foreseen for the Amazon for the year 2100 indicate increases of temperature of 2° C (mild scenario) or even up to 4° C (extreme scenario) (IPCC, 2013). Therefore, during a field trip to the Anavilhanas islands, Rio Negro, 10 local species belonging to the major groups of fishes of the region were selected and the effects of three temperatures (31, 33 and 35° C) on their blood oxygen affinities (P_{50}) *in vitro* were analysed over pHs ranging from 7·0 to 7·8. The aim of this study was to explore the effects of climate changes, specifically increased temperature and decreased pH, on the blood oxygen affinity.

MATERIALS AND METHODS

EXPERIMENTAL SITE AND ANIMALS

All experiments were performed in an air-conditioned room on board the vessel *Ana Clara* from Manaus during an expedition to the Anavilhanas Archipelago of the Rio Negro, *c.* 110 km upstream from Manaus in December 2013. The vessel moored at the ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade) floating base 1 at the entrance of the Prato Lake, Anavilhanas National Park (2° 23′ 41″ S; 60° 55′ 14″ W), for the entire expedition period. All procedures were in compliance with Brazilian national and INPA animal care regulations (Sisbio: 29837-6; CEUA: 047/2012).

A total of 42 animals belonging to 10 species of five orders of fishes of the Amazon were sampled (Table I). Fishes used for this study were caught by INPA fishermen using various types of fishing gears in three locations from the surroundings of the floating base (2° 43′ 054″ S; 60° 44′ 709″ W and 2° 41′ 449″ S; 60° 45′ 705″ W and 2° 41′ 427″ S; 60° 45′ 837″ W) and were: freshwater sting ray *Potamotrygon motoro* (Müller & Henle 1841), silver arowana *Osteoglossum bicirrhosum* (Cuvier 1829), large scale jaraqui *Semaprochilodus insignis* (Jardine 1841), small scale jaraqui *Semaprochilodus taeniurus* (Valenciennes 1821), piranha *Serrasalmus hollandi* Eigenmann 1915, traira *Hoplias malabaricus* (Bloch 1794), piracatinga *Calophysus macropterus* (Lichtenstein 1819), tucunaré *Cichla monoculus* Agassiz 1831, acará roi-roi *Geophagus proximus* (Castelnau 1855) and papa-terra *Satanoperca jurupari* (Heckel 1840). All collected specimens were recovered on board for at least 24 h in large tanks served with through-flow water pumped directly from Rio Negro (temperature 28–31° C, pH 4·0–4·5). Dissolved oxygen in the tanks was kept near saturation level by continuous artificial aeration. Animals were fasted during holding until blood samples were taken.

EXPERIMENTAL MEASUREMENTS

Animals (see Table I for species and number, n) were individually netted from holding tanks and immediately wrapped in wet tissue, bled, measured (total length, L_T cm) and weighed (g). A blood sample (1·0–1·5 ml) was taken from the caudal vein using a heparinized syringe, transferred to Eppendorf tubes and kept on ice. Sampled animals were allowed to recover and then released. The blood samples were used within 1 h for all analyses as described further. Older samples were discarded due to changes in metabolites, in particular erythrocytic phosphates (pers. obs.).

Haematocrit (Ht) was measured in triplicate after centrifuging whole blood in microhaematocrit tubes at 7,826*g* for 5 min. Blood Hb concentration was determined spectrophotometrically in duplicate at 540 nm following conversion to cyanomethaemoglobin, using Drabkin's reagent (Doles Reagentes e Equipamentos para Laboratórios Ltda.; www.doles.com.br), applying an extinction coefficient of 11. Blood glucose levels were determined in duplicate on a glucometer (Accu-Check, Roche; www.accu-chek.com).

Blood-O_2 dissociation curves were individually obtained using a Hemox analyser (TCS Scientific; www.tcssci.com) for all samples. The validity of this method was checked by running human and fish samples (*H. malabaricus* and *O. bicirrhosum*) at full saturation using pure oxygen and dehumidified air. The calculated local partial pressure of oxygen (pO_2), based on the measured temperature, humidity and barometric pressure, was used to calibrate the instrument. Overall, the reproducibility of P_{50} for a given condition was over 95% for the equipment using fish blood.

Full blood-O_2 dissociation curves at pH 7·0, 7·2, 7·4, 7·6 and 7·8 were obtained at 31 and 35° C only for *H. malabaricus* and *O. bicirrhosum*. For all other species, blood P_{50} was determined at pH 7, 7·4 and 7·8 and at 31, 33 and 35° C. For each measurement, 4 ml of 50 mM Tris (Sigma Chem. Co.; www.sigma.com) buffers (pH 7·4, 7·6 and 7·8) or 50 mM Bis-Tris (Sigma Chem. Co.) buffers (pH 7·0 and 7·2), freshly prepared, were used to dilute 50 µl of blood in an appropriate cuvette for all measurements, according to the manufacturer's instructions. Assay temperature was maintained by a Lauda-Brinkmann (model Ecoline E-100, Lauda-Brinkmann; www.lauda-brinkmann.com) water bath connected to the Hemox. Blood deoxygenation was

TABLE I. Analysed fish species with indication of their order, mass, total length (L_T), blood glucose, haematocrit (Ht), haemoglobin concentration and mean cell haemoglobin concentration (MCHC). Data are means ± S.E.

Species	Order	*n*	Mass (g)	L_T (cm)	Glucose (mmol l^{-1})	Ht (%)	Hb (g%)	MCHC (%)
Potamotrygon motoro (stingray)	Rajiformes	1	12 000	90 (diameter)	1·11	24	5·7	23·8
Osteoglossum bicirrhosum (silver arowana)	Osteoglossiformes	5	630 ± 290	44·8 ± 8·4	8·91 ± 0·92	28·5 ± 1·4	4·8 ± 0·5	16·9 ± 2·0
Semaprochilodus insignis (large scale jaraqui)	Characiformes	5	185 ± 40	19·7 ± 1·6	4·36 ± 0·57	39·2 ± 1·3	8·8 ± 1·2	22·8 ± 3·6
Semaprochilodus taeniurus (small scale jaraqui)	Characiformes	6	187 ± 15	20·2 ± 1·3	8·44 ± 1·81	40·5 ± 2·8	7·2 ± 0·4	17·9 ± 0·9
Serrasalmus hollandi (piranha)	Characiformes	6	155 ± 84	16·1 ± 2·1	3·41 ± 0·33	39·0 ± 1·7	10·4 ± 0·3	26·9 ± 1·4
Hoplias malabaricus (traira)	Characiformes	3	450 ± 78	29·5 ± 0·7	2·05 ± 0·11	29·0 ± 4·0	5·8 ± 1·1	20·5 ± 4·5
Calophysus macropterus (piracatinga)	Siluriformes	6	200 ± 67	24·9 ± 3·5	5·44 ± 0·46	34·0 ± 1·9	5·5 ± 0·5	16·2 ± 1·3
Cichla monoculus (tucunaré)	Perciformes	3	370 ± 36	28·0 ± 0·9	6·60 ± 1·44	36·7 ± 2·2	8·2 ± 1·6	22·4 ± 4·7
Geophagus proximus (acará roi-roi)	Perciformes	6	195 ± 56	24·9 ± 3·5	5·91 ± 1·73	32·0 ± 1·6	4·5 ± 0·7	14·0 ± 2·1
Satanoperca jurupari (papa-terra)	Perciformes	4	149 ± 33	18·0 ± 1·6	8·09 ± 0·41	26·3 ± 2·7	4·0 ± 0·6	14·8 ± 0·8

n, number of animals.

 Journal of Fish Biology 2016, doi:10.1111/jfb.13009

obtained by bubbling ultra pure nitrogen (White Martins; www.praxair.com.br). Blood oxygenation was obtained by bubbling dehumidified air using an air pump Inalar (model Compact, Inalar; www.nsam.com.br) connected to a water trap and then to the Hemox analyser. Oxygen dissociation curves were recorded using custom software developed by the Hemox manufacturer and furnished with the instrument.

CALCULATIONS AND STATISTICS

Data are expressed as mean ± S.E. Mean cell haemoglobin concentration (MCHC) was calculated using the formula $100xy^{-1}$, where x = Hb and y = Ht. The significance of the differences of P_{50} mean values over the tested pHs at a given temperature were evaluated by one-way ANOVA followed by multiple comparison Tukey's test, using Sigma Stat software (Systat Software Inc.; https://systatsoftware.com/). The effects of temperature at a given pH were evaluated by one-way ANOVA and subsequent comparisons with the 31° C value by Dunnett's test, using Sigma Stat software (Systat Software Inc.). In all cases, differences were considered statistically significant at $P \leq 0{\cdot}05$. For *H. malabaricus* and *O. bicirrhosum*, P_{50} values were also plotted against wider range of pH (pH 7·0, 7·2, 7·4, 7·6 and 7·8) and the best fitting curve was calculated for each temperature (31 and 35° C), using Sigma Plot 11. The significance of r for each fitted curve was checked against critical values of correlation coefficients (Zar, 1984) and calculated r-values were considered significant at $P \leq 0{\cdot}05$. The Bohr factor (Φ) was calculated as $\Phi = \Delta \log_{10} P_{50}\, \Delta \mathrm{pH}^{-1}$ for the analysed pH range.

RESULTS

FISHES AND BLOOD CHARACTERISTICS

Blood glucose levels varied among the various species (Table I). Average Ht values were relatively homogeneous among the analysed species, ranging only about 1·5 fold from 26·3% in *S. jurupari* to 40·5% in *S. taeniurus* (Table I). In contrast, Hb concentration showed a wider range (2·6 fold) of variations among the analysed species (Table I), varying from 4·0 g% in *S. jurupari* to 10·4 g% in *S. hollandi*. As a result, the MCHC exhibited an intermediate level of variation (1·9 fold) among all analysed species (Table I).

BOHR SHIFT OF *H. MALABARICUS* AND *O. BICIRRHOSUM*

Blood of *H. malabaricus* and *O. bicirrhosum* were analysed over five relevant physiological pHs, allowing calculation of regression curves for pH and $\log_{10} P_{50}$ (Fig. 1). Higher blood-O_2 affinities (lower P_{50}) were present in *H. malabaricus* compared to *O. bicirrhosum* over all the analysed pH range. Both species showed normal Bohr shifts at both analysed temperatures: $\Phi = -0{\cdot}44$ at 31° C and $\Phi = -0{\cdot}26$ at 35° C for *H. malabaricus* and $\Phi = -0{\cdot}54$ at 31° C and $\Phi = -0{\cdot}58$ at 35° C for *O. bicirrhosum*, indicating a greater pH sensitivity in the latter. Note the larger significant effect of temperature (*i.e.* greater separation of the lines) on blood-O_2 affinity of *H. malabaricus* over the entire analysed pH range, while increased temperature had smaller effect on blood O_2 affinity of *O. bicirrhosum* (Fig. 1). At pH 7·4, the blood of *H. malabaricus* had a $P_{50} = 7{\cdot}44 \pm 0{\cdot}76$ mmHg at 31° C and $P_{50} = 11{\cdot}59 \pm 1{\cdot}14$ mmHg at 35° C, which corresponds to a 56% decrease of blood O_2 sensitivity as a result of an increase of 4° C in assay temperature. In contrast, *O. bicirrhosum,* which had a $P_{50} = 11{\cdot}96 \pm 1{\cdot}44$ mmHg at 31° C and $P_{50} = 14{\cdot}63 \pm 1{\cdot}92$ mmHg, exhibited only a 22% decrease of blood O_2 sensitivity for the same temperature change at pH 7·4, a difference of 2·5 fold.

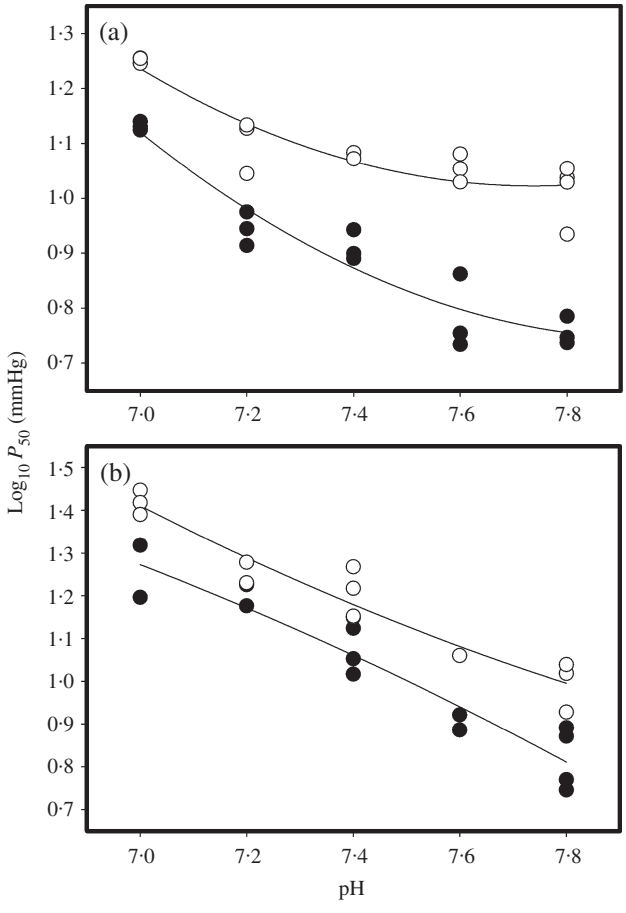

FIG. 1. Bohr plot showing the effect of pH on blood oxygen affinity (P_{50}) at 31° C (●) and 35° C (○) on (a) *Hoplias malabaricus* ($n = 3$) and (b) *Osteoglossum bicirrhosum* ($n = 3$). Note the higher blood O_2 affinities (lower P_{50} values) of *H. malabaricus* at both temperatures ($\Phi = -0{\cdot}44$ at 31° C, $y = 26{\cdot}3976 - 6{\cdot}4426x + 0{\cdot}4045x$; $r^2 = 0{\cdot}92$ and $\Phi = -0{\cdot}26$ at 35° C, $y = 24{\cdot}5232 - 6{\cdot}0744x + 0{\cdot}3925x$; $r^2 = 0{\cdot}82$) compared to *O. bicirrhosum* ($\Phi = -0{\cdot}54$ at 31° C, $y = -4{\cdot}6697 + 2{\cdot}1276x - 0{\cdot}1827x^2$; $r^2 = 0{\cdot}91$ and $\Phi = -0{\cdot}58$ at 35° C, $y = 13{\cdot}1382 - 2{\cdot}7133x + 0{\cdot}1483x^2$; $r^2 = 0{\cdot}92$).

IN VITRO EFFECTS OF TEMPERATURE AND PH ON BLOOD P_{50}

The single analysed blood sample of the *P. motoro* was reactive to both pH and temperature [Fig. 2(a)]. At pH 7·4 and 7·8, as expected, blood O_2 affinity decreased as the temperature increased, but the opposite occurred at pH 7·0. At the lowest temperature, P_{50} almost doubled as the pH decreased from 7·4 to 7·0 suggesting high blood pH sensitivity for this fish species. In the *O. bicirrhosum*, the blood remained sensitive to pH at all three assay temperatures [Fig. 2(b)]. At pH 7·0, increased temperature (35° C) caused a decrease of blood O_2 affinity. Blood O_2 affinity of *O. bicirrhosum* was lower at pH 7·0 and 7·4, compared to pH 7·8 at all analysed temperatures.

Except at pH 7·4 for *H. malabaricus* and *S. taeniurus* [Fig. 3(a), (c)], no effects of increased temperature on P_{50} were observed for a given pH for all analysed species

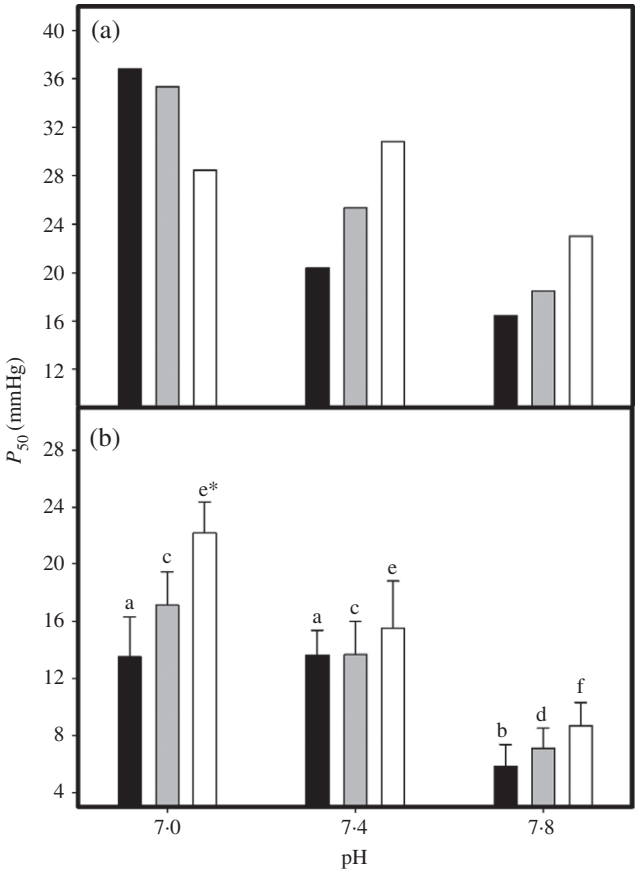

FIG. 2. Effect of pH and temperature (■, 31° C; ▨, 33° C; □, 35° C) on blood oxygen affinity (P_{50}) of (a) *Potamotrygon motoro* ($n = 1$) and (b) *Osteoglossum bicirrhosum* ($n = 5$). Note the unexpected effect of temperature on P_{50} at pH 7·0 of *P. motoro*. Values are means ± S.E. Lower-case letters are used to indicate significant difference of P_{50} over the tested pH range at a given temperature ($P < 0{\cdot}05$). , significant differences of the P_{50} relative to the P_{50} at 31° C at a given pH ($P < 0{\cdot}05$).

of Characiformes (Fig. 3). In contrast, acidification (from pH 7·8 to 7·0) caused the expected decrease of blood O_2 affinity (*i.e.* increased P_{50}) for all analysed species of this group. The two species of the same genus, *S. taeniurus* and *S. insignis*, had similar blood sensitivities under the assay conditions of pH and temperature.

Calophysus macropterus, of the order Siluriformes, showed decreased blood O_2 affinity in response to both decreased pH and increased temperature. Blood O_2 affinity decreased by 50% or more as the pH decreased from 7·8 to 7·0, and by up to 60% as temperature increased from 31 to 35° C at pH 7·4 and 7·8 and by 40% at pH 7·0 (Fig. 4).

The three species of Perciformes, all belonging to the family Cichlidae, exhibited decreased blood sensitivities to decreased pH (Fig. 5). Blood O_2 affinity of the *C. monoculus* also decreased in higher temperature (35° C) at all three pHs analysed. *Satanoperca jurupari* presented a similar situation only in pH 7·8. In contrast, *G.*

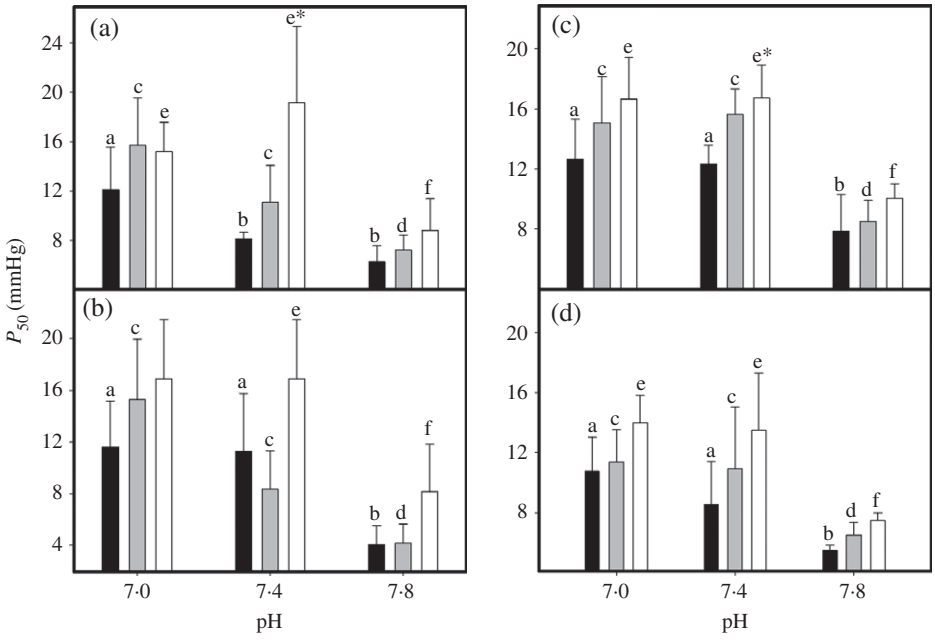

FIG. 3. Effect of pH and temperature (■, 31° C; ▨, 33° C; □, 35° C) on blood oxygen affinity (P_{50}) of (a) *Hoplias malabaricus* ($n = 3$), (b) *Serrasalmus hollandi* ($n = 6$), (c) *Semaprochilodus taeniurus* ($n = 6$) and (d) *Semaprochilodus insignis* ($n = 5$). Note the effect of the highest temperature (35° C) on blood of *H. malabaricus* at pH 7·4. Values are means ± S.E. Lower-case letters are used to indicate significant difference of P_{50} over the tested pH range at a given temperature ($P < 0{\cdot}05$). ⁎, significant differences of P_{50} from 31° C at a given pH ($P < 0{\cdot}05$).

proximus exhibited blood that was insensitive to increased temperature at all three pHs analysed.

DISCUSSION

Stress of studied fish could be rejected since the values observed for glucose, Ht, Hb concentration and MCHC for all 10 species were in the normal range (Table I), compared to previous studies [reviewed by Val & Almeida-Val (1995), Almeida-Val *et al.* (2006) and Brauner & Val (2006)]. The near eight-fold difference observed between the lowest and the highest average glucose levels, respectively, for *P. motoro* and *O. bicirrhosum* (Table I), are consistent with other authors' measurements for a great range of Amazon species. The single value observed for the stingray *P. motoro* was similar to another low value recorded in a different stingray (2·36 mM) (Wood *et al.*, 2002). Variation of glucose levels between species and stages of development have been described for other groups of species (Vijayan & Moon, 1994; Iwama *et al.*, 2004). Increased levels of glucose have been associated with stress as a consequence of mobilization of stress hormones such as catecholamines and cortisol (Iwama *et al.*, 1999*a*, *b*). However, plasma glucose levels should not be taken as the only indicator of stress; it should be considered in conjunction with other indicators (Martínez-Porchas *et al.*, 2009).

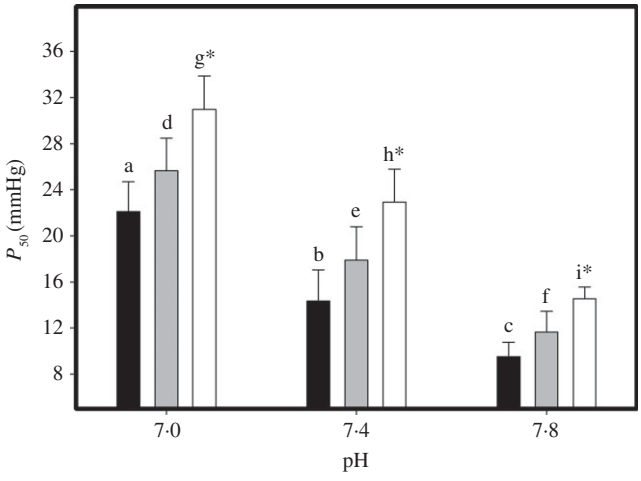

FIG. 4. Effect of pH and temperature (■, 31° C; ▨, 33° C; □, 35° C) on blood oxygen affinity (P_{50}) of *Calophysus macropterus* ($n = 6$). Note that the highest tested temperature caused a significant decrease of blood oxygen sensitivity at all three pHs. Values are means ± S.E. Lower-case letters are used to indicate significant difference of P_{50} over the tested pH range at a given temperature ($P < 0{\cdot}05$). *, significant differences of P_{50} from 31° C at a given pH ($P < 0{\cdot}05$).

Previous studies showed that Ht and Hb concentration in 25 fish species of the Amazon, which had been allowed to recover following capture from wild, ranged from 21 to 35·5% and from 5·3 to 10·6 g%, respectively (Marcon *et al.*, 1999). As blood O_2 affinity is highly influenced by changes of intra-erythrocytic phosphates (Val, 2000; Mairbäurl & Weber, 2012), and these are rapidly adjusted according to stress condition (Val *et al.*, 2015), unstressed animals are needed to test the effects *in vitro* of temperature and pH.

Overall, increased temperatures caused a general decrease of blood O_2 affinity of analysed species. As these fish cannot regulate body temperature and regularly face low oxygen environments, any decrease of blood O_2 affinity would represent a significant constraint. This constraint is further increased at low pH. The ability of these species to improve behavioural and physiological adjustments over time, so as to reduce the effects of increased temperatures, increased CO_2 and lowered pH, was not analysed here. Conceivably, such adjustments could help them to reduce or even offset the effects of these environmental changes anticipated by IPCC for the year 2100.

Hoplias malabaricus and *O. bicirrhosum* had contrasting blood O_2 affinities over the pH and temperature range tested (Fig. 1). The blood of *H. malabaricus* was 2·5 fold more sensitive to increased temperature at pH 7·4 than the blood of *O. bicirrhosum* at the same pH. Similarly, the Bohr factor in *H. malabaricus* was higher by 23% at 31° C and by 123% at 35° C than the values observed for *O. bicirrhosum*. It has been shown that blood O_2 affinity varies considerably among species (Brauner & Randall, 1998), mainly related to their specific multiple Hb components that have different functional properties (Jensen, 2004). This may be the case for these two species: while the *H. malabaricus* has five anodic Hb components, only two anodic components were detected for the blood of *O. bicirrhosum* on agar-starch gel electrophoresis (Val *et al.*, 1987). Reischl (1976) and Fyhn *et al.* (1979) reported similar Hb patterns for these species using different electrophoresis systems.

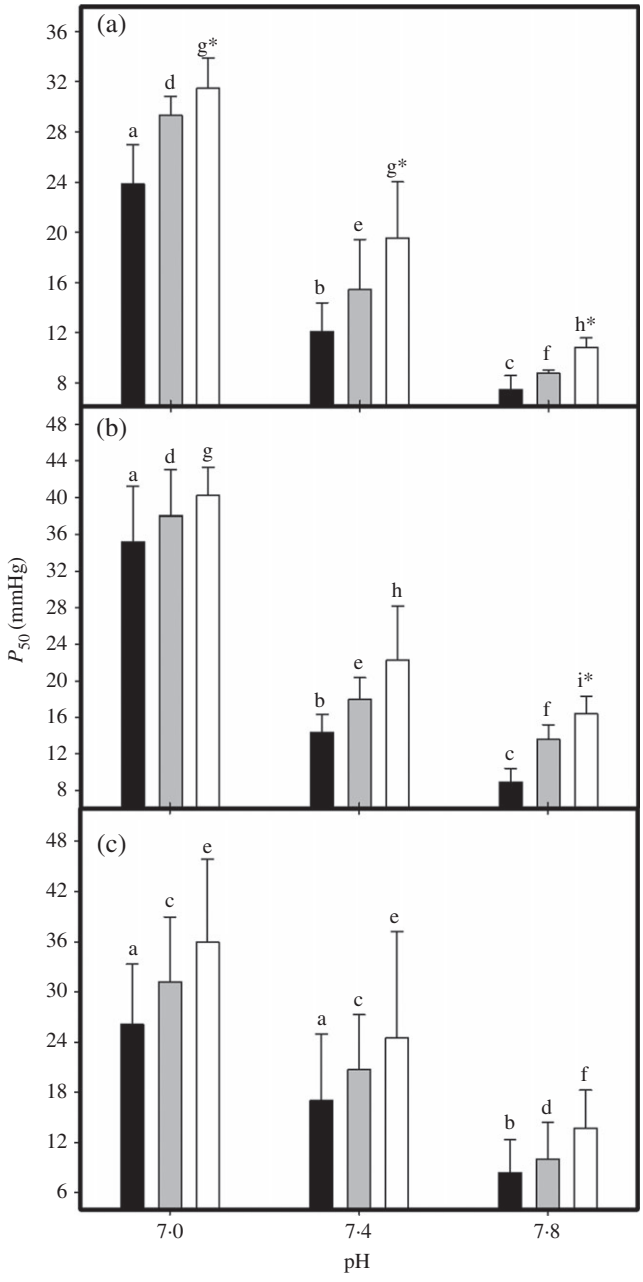

FIG. 5. Effect of pH and temperature (■, 31° C; ▨, 33° C; □, 35° C) on blood oxygen affinity (P_{50}) of (a) *Cichla monoculus* ($n = 3$), (b) *Satanoperca jurupari* ($n = 4$) and (c) *Geophagus proximus* ($n = 6$). Note that the highest tested temperature caused a significant decrease of blood O_2 sensitivity of *C. monoculus* at all three pHs, and of *G. jurupari* at pH 7·8. Values are means ± S.E. Lower-case letters are used to indicate significant difference of P_{50} over the tested pH range at a given temperature ($P < 0{\cdot}05$). ⁕, significant differences of P_{50} from 31° C at a given pH ($P < 0{\cdot}05$).

The marked difference of Bohr factor of the blood of these two species tested at higher temperature (35° C) is mainly explained by the lowered affinity (higher P_{50}) above pH 7·4 in *H. malabaricus*, compared to *O. bicirrhosum*. The blood of this latter species had, as expected, a lowered affinity at the higher temperature (35° C) at all tested pHs, but with no interaction with pH, contrasting with the blood of *H. malabaricus* (Fig. 1).

Hb-O_2 affinity decreases with rising temperature basically due to the exothermic nature of the oxygenation of haem groups. An effect of temperature on the erythrocytic micro-environment, basically on levels of allosteric effectors such as chloride, lactate and organic phosphates, would reduce or even offset decreased Hb-O_2 affinity at higher temperatures (Wood *et al.*, 1978; Weber & Campbell, 2010). So, the stripped haemoglobin of both *H. malabaricus* and *O. bicirrhosum* would show even lower Bohr factors than the values observed for their whole blood. However, in contrast, their blood O_2 affinities would have been adjusted *in vivo* if the animals were acclimated to higher temperatures either by adjusting the concentration of their multiple haemoglobins, such as observed for rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Weber *et al.*, 1976) or their intra-erythrocytic phosphate levels, such as observed for the Australian black fish *Gadopsis marmoratus* Richardson 1848 (Dobson & Baldwin, 1982).

These data suggest that the blood of *H. malabaricus* would be more sensitive than the blood of *O. bicirrhosum* to the 4° C water warming likely to occur under the extreme climate change scenarios for the year 2100 (IPCC, 2013). It is worthwhile pointing out that the effect of temperature in reducing Hb-O_2 affinity is generally considered as an advantage as it results in an increased O_2 unloading to respiring tissues. However, this would represent a constraint for fish facing warmer hypoxic waters, when a decreased Hb-O_2 affinity caused by increased temperature would reduce oxygen binding at the gills and hence, oxygen transfer to respiring tissues.

Are the differences observed for *H. malabaricus* and *O. bicirrhosum* also observed for other fish species of the Amazon? Are they like either *H. malabaricus* or *O. bicirrhosum*? Do the responses follow the biological diversity of the ecosystem? To address these questions, eight other species belonging to diverse phylogenetic groups were then analysed (Table I).

Out of the 10 species analysed, a single large specimen of *P. motoro* (Rajiformes) (the only one captured) was the only freshwater cartilaginous fish analysed and showed a very specific profile [Fig. 2(a)]: the blood O_2 affinity at pH 7·0 increased at higher temperature, contrasting with the effects observed at alkaline pHs (7·4 and 7·8). At 35° C, P_{50} at pH 7 is slightly lower than at pH 7·4. If statistically confirmed, this finding contrasts with nearly all other vertebrates that have a decreased Hb-O_2 affinity at higher temperatures, such as several species analysed here, including an ancient bony fish, the osteoglossid *O. bicirrhosum* [Fig. 2(b)]. Well-known examples of reversed temperature dependence of blood O_2 affinity (increased temperature causing an increase of Hb-O_2 affinity) additionally include tuna species that developed what is termed 'regional endothermy' [reviewed by Clark *et al.* (2010)]. Additional analysis of purified haemoglobins and whole blood of freshwater stingrays is needed to confirm the unusual effect of temperature on the blood O_2 affinity of *P. motoro* at pH 7·0.

As expected, the four species of characids showed an increase of P_{50} as the pH decreased from 7·8 to 7·0, a change that will facilitate oxygen unloading to respiring tissues. This is almost a universal response of fish blood to pH changes and the respective underlying mechanisms are known and accepted (Jensen, 2004). Concurrently, these four species showed a blood O_2 affinity that slightly decreased (increased

P_{50}) over the higher tested temperatures, although most of the P_{50} values differences were not significant (Fig. 3). The blood of *H. malabaricus* [Fig. 3(a)] and *S. insignis* [Fig. 3(d)], at pH 7·4, were the two exceptions. Overall, the analyses of these species suggest that their haemoglobins are nearly insensitive to minor increases in temperature. Similar situations have been observed for other fish species analysed by Kaufman *et al.* (2006). Although this may not represent a constraint for animals to upload oxygen when facing deep hypoxia in warmer waters, a combination of behavioural, physiological and biochemical mechanisms can be directed to offset these minor effects *in vivo*. The regulation of Hb isoforms having different functional properties, as well as adjustments of the major intra-erythrocytic modulators such as ATP and GTP can be directed towards reducing environmental constraints (Val *et al.*, 1984, 2015; Monteiro *et al.*, 1987; Rutjes *et al.*, 2007). An interesting example was reported by Weber *et al.* (2010) studying billfish, a group of heterothermic predators. As observed for the characids analysed in this study (Val *et al.*, 1987), billfish express multiple Hb isoforms with pronounced sensitivities of Hb-O_2 affinities to pH and insensitivity to temperature in the presence of ATP (Weber *et al.*, 2010). It is noteworthy that the analysed characid fish species routinely undergo severe exercise either as predators, short-distance, fast swimmers (*H. malabaricus* and *S. hollandi*) or as long-distance swimmers (*S. insignis* and *S. taeniurus*), but do not show regional heterothermy.

Calophysus macropterus was the only analysed catfish species of the Order Siluriformes. Compared to characids, catfishes tend to have a lower average number of Hb isoforms, 5·2 against 2·5, respectively (Fyhn *et al.*, 1979; Galdames-Portus *et al.*, 1982; Val *et al.*, 1987), so it is possible that this group would have proportionally reduced options to offset the effects of environmental and physiological constraints on blood O_2 binding and unloading. Specifically, *C. macropterus* had very typical decreased blood O_2 affinities over the pH range tested (pH 7·8–7·0) (Fig. 4), showing a relatively high Bohr factor of $\phi = -0{\cdot}46$ at 31° C, compared to other fish species of the Amazon belonging to this group (Powers *et al.*, 1979). These authors analysed 18 species belonging to seven families of the Order Siluriformes and reported Bohr factors for whole blood ranging between $\Phi = -0{\cdot}10$ and $\Phi = -0{\cdot}60$, with 14 species having $\Phi < -0{\cdot}40$. So, *C. macropterus* is among the catfishes with the highest values.

The increases in P_{50} were not significant in all three tested pHs for blood samples of *C. macropterus* assayed at 33° C. At 35° C, however, significantly increased P_{50} values were observed for all three tested pH (Fig. 4), resulting in a Bohr factor of $\Phi = -0{\cdot}41$, very close to that observed at 31° C. The decreased blood O_2 affinity that would facilitate oxygen unloading to respiring tissues would, however, represent a constraint for this species against oxygen loading in the hypoxic warmer waters likely to occur in certain areas of the Amazon within the climate change scenarios anticipated for the near future. *Calophysus macropterus* is also known as the vulture catfish and is found in high density scavenging food, in particular offal, thrown by humans into the river, when they remain close to water surface. Otherwise, they stay at the bottom where relatively lower temperatures are found. Therefore, increased water temperature would affect blood O_2 binding in this fish species, particularly during feeding periods.

A very typical and similar pattern was also observed for the three species of cichlids analysed. All three species showed increased P_{50} values as pH decreased over the range tested, as observed for the catfish *C. macropterus* (Fig. 4). The cichlids are very diverse and are the most derived fish group of the Amazon and connected regions, belonging to the Order Perciformes (Nelson, 1994; Val & Almeida-Val, 1995; Kullander & Ferreira,

2006). Many cichlid species are territorial, with the female collecting the fertilized eggs in the mouth and the male displaying brood-care behaviour. Some species make nests during the breeding period and despite being gregarious, they become very aggressive at this time, as is the case for *C. monoculus* (Molfgang & Schierwater, 1988; Yamamoto *et al.*, 1999). Certainly, a reliable oxygen transfer system is needed for these diverse biological activities.

As observed for the catfish, all three species of cichlids showed minor decreases of blood O_2 affinities as the assay temperature was increased to 33° C at all three tested pHs. When the assay temperature was increased to 35° C, however, the decrease of blood O_2 affinities was significant at all three tested pHs for *C. monoculus* and at pH 7·8 for *S. jurupari*. No significant effect of assay temperature of 35° C was observed for the blood of *G. proximus*. These differences, even within closely related species belonging to the same group (family and order), can be related to their behaviour that differs among the three species: *G. proximus* is omnivorous, eating seeds, fruits, small crustaceans and insect larvae, and both male and female are mouth brooders. These characteristics allow them to move away from warmer and hypoxic water; *S. jurupari*, although omnivorous too, additionally feeds on small fishes, a diet that requires some exercise, and the eggs are deposited over twigs or gravels; only after 24 h are the fertilized eggs collected by the female and kept in the mouth. This lifestyle may expose the parents to episodes of hypoxia (Ferreira *et al.*, 1998). So, *S. jurupari* must be prepared to unload oxygen to respiring tissues during exercise when it could get exposed to warmer waters, as well as to uptake oxygen from unfavourable hypoxic environments. These requirements are safeguarded by its very large Bohr factor and the significant effect of increased temperature on blood P_{50} at 35° C at pH 7·8 [Fig. 5(b)]. Contrasting with these two species, *C. monoculus* is carnivorous, feeding mainly on fishes, and it inhabits mainly floodplain lakes between dead twigs and flooded tree branches, as well as the shorelines of black and white waters (Kullander & Ferreira, 2006). Warmer waters and low oxygen are often found in these shallow environments in the Amazon and the blood O_2 properties of *C. monoculus* [Fig. 5(a)] appear to be designed to safeguard oxygen delivery to tissues under these unfavourable conditions.

In summary, this study showed that the increases of temperature foreseen by IPCC for coming years cause a decrease of Hb-O_2 affinities of different intensities, relative to the highest average temperature of the Rio Negro, for the species analysed in all three assayed pHs. As expected, blood acidification also causes a decrease of Hb-O_2 affinities for all species analysed. The interspecific differences are better related to the habitat and biological characteristics of the species than to their systematic positions. Finally, the species having more thermally insensitive blood (*S. hollandi*, *S. insignis* and *G. proximus*) would cope better with warmer environments.

Financial support from INCT ADAPTA–CNPq/FAPEAM and Ciência sem Fronteiras is gratefully acknowledged. A.L.V. and V.M.F.A.-V. are recipients of research fellowships from the Brazilian CNPq. C.M.W. was supported by the Canada Research Chair Program and is the recipient of a fellowship from the Science Without Borders Program (CNPq-Brazil).

References

Almeida-Val, V. M. F., Chippari-Gomes, A. R. & Lopes, N. P. (2006). Metabolic and physiological adjustments to low oxygen and high temperature in fish of the Amazon. *The Physiology of Tropical Fishes* (Val, A. L., Almeida-Val, V. M. F. & Randall, D. J.), 443–500. London: Elsevier.

Brauner, C. J. & Randall, D. J. (1998). The linkage between oxygen and carbon dioxide transport. *Fish Physiology,* 17 (Perry, S. F. & Tufts, B. L.), 283–319. San Diego, CA: Academic Press.

Brauner, C. J. & Val, A. L. (2006). Oxygen transfer. *The Physiology of Tropical Fish* (Val, A. L., Almeida-Val, V. M. F. & Randall, D. J.), 277–306. San Diego, CA: Elsevier/Academic Press.

Caraballo, P., Forsberg, B. R., Almeida, F. F. & Leite, R. G. (2014). Diel patterns of temperature, conductivity and dissolved oxygen in an Amazon floodplain lake: description of a friagem phenomenon. *Acta Limnologica Brasiliensia* **26,** 318–331.

Clark, T. D., Rummer, J. L., Sepulveda, C. A., Farrell, A. P. & Brauner, C. J. (2010). Reduced and reversed temperature dependence of blood oxygenation in an ectothermic scombrid fish: implications for the evolution of regional heterothermy? *Journal of Comparative Physiology B* **180,** 73–82.

Dobson, G. P. & Baldwin, J. (1982). Regulation of blood oxygen affinity in the Australian blackfish *Gadopsis marmoratus*. II. Thermal acclimation. *Journal of Experimental Biology* **99,** 245–254.

Ferreira, E. J. G., Zuanon, J. A. S. & Santos, G. M. (1998). *Peixes comerciais do Médio Amazonas – Região de Santarém, Pará*. Brasília: IBAMA.

Fyhn, U. E. H., Fyhn, H. J., Davis, B. J., Powers, D. A., Fink, W. L. & Garlick, R. L. (1979). Hemoglobin heterogeneity in Amazonian fishes. *Comparative Biochemistry and Physiology* **62A**, 39–66.

Galdames-Portus, M. I. G., Donald, E. L. & Focesi, A. Jr. (1982). Hemoglobinas em silurídeos da Amazônia Central. I. Análise eletroforética dos hemolisados. *Acta Amazonica* **12,** 707–711.

Holt, R. E. & Jorgensen, C. (2015). Climate change in fish: effects of respiratory constraints on optimal life history and behaviour. *Biology Letters* **11,** 20141032. http://dx.doi.org/10.1098/rsbl.2014.1032.

IPCC (2013). Climate change 2013: the physical science basis. *Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V. & Midgley, P. M.), 1535. Cambridge: IPCC.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B. & Ackerman, P. (1999*a*). Heat shock proteins and physiological stress in fish. *American Zoologist* **39,** 901–909.

Iwama, G. K., Vijayan, M. M. & Morgan, J. D. (1999*b*). The stress response in fish. *Ichthyology Recent Research Advances* (Saksena, D. N.), 47–57. Enfield, NH: Science Publishers, Inc.

Iwama, G. K., Afonso, L. O. B., Todgham, A. E., Ackerman, P. & Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? *Journal of Experimental Biology* **207,** 15–19.

Jensen, F. B. (2004). Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O_2 and CO_2 transport. *Acta Physiologica Scandinavica* **182,** 215–227.

Kaufman, R. C., Houck, A. G. & Cech, J. J. Jr. (2006). Effects of temperature and carbon dioxide on green sturgeon blood-oxygen equilibria. *Environmental Biology of Fishes* **76,** 119–127.

Kullander, S. O. & Ferreira, E. J. G. (2006). A review of the South American cichlid genus *Cichla*, with descriptions of nine new species. *Ichthyological Exploration of Freshwaters* **17,** 289–398.

Mairbäurl, H. & Weber, R. E. (2012). Oxygen transport by hemoglobin. *Comprehensive Physiology* **2,** 1463–1489.

Marcon, J. L., Chagas, E. C., Kavassaki, J. M. & Val, A. L. (1999). Intraerythrocytic phosphates in 25 fish species of the Amazon: GTP as a key factor in the regulation of Hb-O_2 affinity. *Biology of Tropical Fish* (Val, A. L. & Alameida-Val, V. M. F.), 229–240. Manaus: INPA.

Martínez-Porchas, M., Martínez-Cordova, L. R. & Ramos-Henriquez, R. (2009). Cortisol and glucose: reliable indicators of fish stress? *Pan-American Journal of Aquatic Sciences* **4,** 158–178.

McCormack, T. J., McKinlay, R. S., Roubach, R., Almeida-Val, V. M. F., Val, A. L. & Driedzic, W. R. (2003). Changes in ventilation, metabolism, and behaviour, but not bradycardia,

contribute to hypoxia survival in two species of Amazonian armoured catfish. *Canadian Journal of Zoology* **81,** 272–280.
Molfgang, M. & Schierwater, B. (1988). Energy expenditure for mouthbrooding in a cichlid fish. *Behavioral Ecology and Sociobiology* **22,** 161–164.
Monteiro, P. J. C., Val, A. L. & Almeida-Val, V. M. F. (1987). Biological aspects of Amazonian fishes. Hemoglobin, hematology, intraerythrocytic phosphates and whole blood Bohr effect of *Mylossoma duriventris*. *Canadian Journal of Zoology* **65,** 1805–1811.
Nelson, J. S. (1994). *Fishes of the World*. New York, NY: John Wiley & Sons.
Nobre, A. D. (2014). *O Futuro Climático da Amazônia. Relatório de Avaliação Científica*. São Paulo: ARA.
Powers, D. A., Fyhn, H. J., Fyhn, U. E. H., Martin, J. P., Garlick, R. L. & Wood, S. C. (1979). A comparative study of the oxygen equilibria of blood from 40 genera of Amazon fishes. *Comparative Biochemistry and Physiology* **62A**, 67–85.
Reischl, E. (1976). The hemoglobins of the fresh-water teleost *Hoplias malabarica* (Bloch, 1794): heterogeneity and polymerization. *Comparative Biochemistry and Physiology* **55B**, 255–257.
Rummer, J. L., Couturier, C. S., Stecyk, J. A. W., Gardiner, N. M., Kinch, J. P., Nilsson, G. E. & Munday, P. L. (2014). Life on the edge – thermal optima for aerobic scope of equatorial reef fishes are close to current day temperatures. *Global Change Biology* **20,** 1055–1066.
Rutjes, H. A., Nieveen, M. C., Weber, R. E., Witte, F. & Van den Thillart, G. E. E. J. M. (2007). Multiple strategies of Lake Victoria cichlids to cope with lifelong hypoxia include hemoglobin switching. *American Journal of Physiology* **293,** R1376–R1383.
Sioli, H. (1984). *The Amazon. Limnology and Landscape Ecology of a Mighty Tropical River and Its Basin*. Dordrecht: Dr W. Junk Publishers.
Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *Journal of Experimental Biology* **213,** 912–920.
Tewksbury, J. J., Huey, R. B. & Deutsch, C. A. (2008). Putting the heat on tropical animals. *Science* **320,** 1296–1297.
Val, A. L. (2000). Organic phosphates in the red blood cells of fish. *Comparative Biochemistry and Physiology* **125A**, 417–435.
Val, A. L. & Almeida-Val, V. M. F. (1995). *Fishes of the Amazon and Their Environments. Physiological and Biochemical Features*. Heidelberg: Springer-Verlag.
Val, A. L., Almeida-Val, V. M. F., Schwantes, A. R. & Schwantes, M. L. B. (1984). Biological aspects of Amazonian fishes I. Red blood cell phosphates of schooling fishes (genus *Semaprochilodus*: Prochilodontidae). *Comparative Biochemistry and Physiology* **78,** 215–217.
Val, A. L., Almeida-Val, V. M. F. & Monteiro, P. J. (1987). Aspectos biológicos de peixes amazônicos. IV. Padrões eletroforéticos de hemoglobinas de 22 espécies coletadas na ilha da Marchantaria (Manaus-AM). *Acta Amazonica* **16–17**, 125–134.
Val, A. L., Gomes, K. R. M. & Almeida-Val, V. M. F. (2015). Rapid regulation of blood parameters under acute hypoxia in the Amazonian fish *Prochilodus nigricans*. *Comparative Biochemistry and Physiology A,* **184,** 125–131.
Vijayan, M. M. & Moon, T. W. (1994). The stress response and the plasma disappearance of corticosteroid and glucose in a marine teleost, the sea raven. *Canadian Journal of Zoology* **72,** 379–386.
Weber, R. E. & Campbell, K. L. (2010). Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. *Acta Physiologica* **202,** 549–562.
Weber, R. E., Wood, S. C. & Lomholt, J. P. (1976). Temperature acclimation and oxygen-binding properties of blood and multiple haemoglobins of rainbow trout. *Journal of Experimental Biology* **65,** 333–345.
Weber, R., Fago, A., Val, A. L., Bang, A., Van Hauwaeert, M. L., De Wilde, S., Zal, F. & Moens, L. (2000). Isohemoglobin differentiation in the biomodal-breathing Amazon catfish *Hoplosternum littorale*. *Journal of Biological Chemistry* **275,** 17297–17305.
Weber, R. E., Campbell, K. L., Fago, A., Malte, H. & Jensen, F. B. (2010). ATP-induced temperature independence of hemoglobin-O2 affinity in heterothermic billfish. *Journal of Experimental Biology* **213,** 1579–1585.

Wood, S. C., Lykkeboe, G., Johansen, K., Weber, R. E. & Maloiv, G. M. O. (1978). Temperature acclimation in the pancake tortoise, *Malacochersus tornieri*: metabolic rate, blood pH, oxygen affinity and red cell organic phosphates. *Comparative Biochemistry and Physiology* **59A**, 155–160.

Wood, C. M., Matsuo, A. Y. O., Gonzalez, R. J., Wilson, R. W., Patrick, M. L. & Val, A. L. (2002). Mechanisms of ion transport in *Potamotrygon*, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro. *Journal of Experimental Biology* **205,** 3039–3054.

Wood, C. M., Iftikar, F., Scott, G. R., de Boeck, G., Sloman, K., Matey, V., Valdez Domingos, F. X., Duarte, R. M., Almeida-Val, V. M. F. & Val, A. L. (2009). Regulation of gill transcellular permeability and renal function during acute hypoxia in the Amazonian oscar (*Astronotus ocellatus*): new angles to the osmorespiratory compromise. *Journal of Experimental Biology* **212,** 1949–1964.

Yamamoto, M. E., Chellappa, S., Cacho, M. S. R. F. & Huntingford, F. A. (1999). Mate guarding in an Amazonian cichlid, *Pterophyllum scalare*. *Journal of Fish Biology* **55,** 888–891.

Zar, J. H. (1984). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall.

