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Red blood cell adrenergic responses in Amazonian teleosts

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Erythrocytes from Amazonian teleosts of the Rio Negro were surveyed for the presence of adrenergically mediated Na^+/H^+ exchange. Washed red blood cells (RBCs) incubated in HEPES-buffered Cortland saline were stimulated with 10^{-4} M L-adrenaline. The adrenergic response was clearly present in two characids, the tambaqui *Colossoma macropomom* and the jaraqui *Semaprochilodus insignis*, as demonstrated by a decrease in the pH_e–pH_i gradient across the RBC membrane, an uptake of Na^+ from the extracellular medium, and RBC swelling. The latter was signalled by increased mean corpuscular volume (MCV) and decreased mean corpuscular haemoglobin concentration (MCHC). The response did not occur in two other characids, the black piranha *Serrasalmus rhombeus* and the aracu *Leporinus fasciatus* or in two silurid catfish, the piranambu *Pinirampus pirinampu* and the acari-bodo, armoured catfish, *Pterygoplichthys multiradiatus*. In acari-bodo, the Na^+/H^+ exchange response was similarly lacking under anoxic conditions. Oxygenated/deoxygenated comparisons revealed the presence of a marked Root effect in jaraqui and its absence in acari-bodo. GTP dominated over ATP as the major intracellular phosphate in all six species. There were no significant changes in any nucleoside phosphate (ATP, ADP, AMP, GTP, GDP, or GMP) in response to adrenaline in any species.

Key words: red blood cell; intracellular pH; adrenaline; Na^+/H^+ exchange; ATP; GTP; Characiformes; Siluriformes.

INTRODUCTION

As first documented by Nikinmaa (1982, 1983), the mobilization of catecholamines into the blood of trout *Oncorhynchus mykiss* (Walbaum) during hypoxia, severe exercise, or generalized stress causes red cell swelling, a decrease in pH gradient across the erythrocyte cell membrane, and a decrease in intracellular nucleoside triphosphate levels (NTP). Extensive studies, almost entirely on members of the genus *Oncorhynchus* (Boutilier & Ferguson, 1989; Motais *et al.*, 1992; Nikinmaa, 1992), have demonstrated that the response reflects stimulation of β-adrenoreceptors on the erythrocyte membrane, consequent activation of adenylate cyclase, production of c-AMP as an intracellular messenger, and activation of cell surface Na^+/H^+ antiporters. The entry of Na^+ along its electrochemical gradient causes a net extrusion of H^+ ions, thereby lowering extracellular pH_e and raising intracellular pH_i. Rising intracellular [Na^+] activates Na^+,K^+ATPase, and erythrocytic [NTP] is reset to a lower level. Cl^- also enters via band 3, driven by the accompanying rise in intracellular

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[HCO_3^-], and an osmotic swelling ensues. The adaptive significance of the response is that the rise in pH_i, the reduction in [NTP], and the dilution of haemoglobin and its allosteric modifiers by cell swelling all help improve blood O_2 capacity and affinity at times of hypoxia and extracellular acidosis.

At present, the generality of the phenomenon is unclear; more than 90% of published reports have been on one species, the rainbow trout. While the mechanism is present in the red cells of all members of the Salmoniformes examined to date, only a few species from other orders have been investigated. In the only three elasmobranchs tested, the response is absent (Tufts & Randall, 1989; Wood *et al.*, 1994). Within the teleosts, the response is absent in two members of the Anguilliformes [the eels *Anguilla rostrata* Lesueur (Hyde & Perry, 1990) and *A. anguilla* L. (Gallardo Romero *et al.*, 1996], of marginal significance in one member of the Pleuronectiformes [the starry flounder *Platichthys stelatus* Pallas (Milligan & Wood, 1987; Wood & Milligan, 1987)] but prominent in another [the European flounder *Pleuronectes flesus* L. (Thoroed *et al.*, 1995)] and quite small in two members of the Cypriniformes [the carp *Cyprinus carpio* L. (Salama & Nikinmaa, 1988, 1989) and the tench *Tinca tinca* L. (Jensen, 1987)]. However the mechanism appears to be fully expressed in one member of the Gadiformes [the Atlantic cod *Gadus morhua* L. (Berenbrink & Bridges, 1994)]; two members of the Perciformes [the striped bass *Morone saxatilis* Walbaum (Nikinmaa & Huestis, 1984; Nikinmaa *et al.*, 1984), the pikeperch *Stizostedion lucioperca* L. (Salama & Nikinmaa, 1989); and the salmoniform pike *Esox lucius* L. (Cossins & Kilbey, 1991)].

The richest ichthyofauna in the world is found in the Amazon basin, representing 20% of all extant freshwater species (Val & Almeida-Val, 1995). Many of these are highly active, and many are exposed frequently to severe hypoxia and severely low pH, especially in the dilute acidic waters of the Rio Negro. However, at present, nothing is known about red cell adrenergic responsiveness in Amazonian teleosts. A field trip to the Anavilhanas Archipelago on the Rio Negro provided an opportunity to survey red cell responses in six Amazonian species of two orders (Characiformes, Siluriformes) which have never been examined before. The erythrocytic transmembrane pH gradient, cell swelling, Na^+ uptake, and intracellular nucleoside phosphate (ATP, ADP, AMP, GTP, GDP, GMP) levels were all examined as possible indices of adrenergic responses.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Experiments were performed during December 1995 on board the INPA Research Vessel *Amanaí II* moored in the Prato Lake, Anavilhanas Archipelago on the Rio Negro (60°45′S, 2°43′W) approximately 75 km upstream from the city of Manaus. The species studied were: Characiformes—(i) tambaqui, *Colossoma macropomom* Cuvier, (ii) black piranha, *Serrasalmus rhombeus* L., (iii) jaraqui, *Semaprochilodus insignis* Schomburgk, (iv) aracu, *Leporinus fasciatus* Bloch; Siluriformes—(v) piranambu, *Pinirampus pirinampu* Spix, and (vi) acari-bodo (armoured catfish), *Pterygoplichthys multiradiatus* Hancock. All fish were in the weight range 180–800 g. With the exception of tambaqui, the species studied were obtained locally by seine net from the Rio Negro

and then held for 8–24 h to recover in large fibreglass tanks supplied on a flow-through basis with water pumped directly from the river. Measured water chemistry was $Na^+ = 52\ \mu mol\ l^{-1}$, $K^+ = 27\ \mu mol\ l^{-1}$, $Ca^{2+} = 10\ \mu mol\ l^{-1}$, $Cl^- = 55\ \mu mol\ l^{-1}$, pH = 5·5, temperature = 30° C. The tambaqui were obtained from commercial aquaculture in Manaus, transported on board the R.V. *Amanaí II* to the Anavilhanas Archipelago, and held under the same conditions for 6 days prior to experiment. This species does occur naturally in the Rio Negro (Val & Almeida-Val, 1995).

EXPERIMENTAL PROTOCOLS

Fish were netted quickly from the holding tank, bled (2–3 ml) via caudal puncture into a syringe heparinized with 500 i.u. lithium heparin, and then generally returned to the wild. The blood was kept on ice, then centrifuged gently (500 *g* for 5 min). Plasma and white cells were discarded, and the remaining red blood cells were washed twice in HEPES-buffered Cortland saline (see below) before overnight storage in the dark in a refrigerator at 4° C. This period allowed recovery from possible adrenergic stimulation/desensitization associated with the stress of blood sampling (Bourne & Cossins, 1982; Walsh *et al*., 1998).

As there were no facilities for working with mixed gases on board the research vessel, all experiments were performed in HEPES-buffered, HCO_3^--free saline (Nikinmaa & Huestis, 1984; Gallardo Romero *et al*., 1996). This saline consisted of Cortland saline for freshwater teleosts (Wolf, 1963) in which the standard 11·9 mmol l^{-1} $NaHCO_3$ was replaced with 10 mM HEPES sodium salt, and the final working pH adjusted to 7·4 with 0·1 N HCl. The natural catecholamine adrenaline was used as the experimental adrenergic agonist. Stock solutions of 10^{-3} M citric acid (a catecholamine preservative) and 10^{-2} M L-adrenaline bitartrate (Sigma) in 10^{-3} M citric acid were made fresh daily and stored in light-tight bottles.

After overnight storage at 4° C, the cells were washed a third time and resuspended to a haematocrit of approximately 20% in this same saline (chilled). Paired aliquots of 1 ml for each individual fish were then prepared in 1·5-ml plastic centrifuge tubes, and allowed to warm to experimental (air) temperature (28–30° C) for 2 h. During this time the suspensions were mixed frequently by inversion to ensure air equilibration. At time 0, 10 µl of 10^{-3} M citric acid were added to the control sample, and 10 µl of 10^{-2} M L-adrenaline bitartrate (Sigma) in 10^{-3} M citric acid were added to the experimental sample, yielding a final adrenaline concentration of 10^{-4} M in the latter. The aliquots were mixed thoroughly and incubated for 15 min prior to sampling.

At sampling, the suspension was mixed thoroughly again, and duplicate aliquots removed for the measurement of haemoglobin concentration (Hb, cyanmethaemoglobin method), haematocrit (Ht, centrifugation at 5000 *g* for 5 min), red blood cell (RBC) counts (Neubauer chamber) and extracellular pH_e (Radiometer E5021 microcapillary electrode system plus PHM 71 Mk2 acid-base analyser). In addition, for measurement of RBC phosphates, 100 µl was immediately deproteinized in 200 µl ice-cold 8% perchloric acid, spun briefly, the supernatant neutralized with 6 M KOH, and stored at −70° C for later chromatographic analysis. High pressure liquid chromatography (HPLC) was used to measure adenylates (ATP, ADP and AMP) and guanylates (GTP, GDP, and GMP). The procedure was carried out using an LKB 2152 HPLC controller and 2150 titanium pump coupled to a 2220 recording integrator. The separation was performed on an Aquapore AX-300 7-µm weak anion exchanger eluting at 2 ml min^{-1} according to Val *et al*. (1994). The remaining sample was centrifuged immediately at 10 000 *g* for 2 min, the supernatant drawn off for later determination of extracellular Na^+ concentration (CELM flame photometer, model FC108), and the red blood cell pellet frozen in liquid N_2 for the measurement of RBC intracellular pH_i (Radiometer E5021).

As there were restricted facilities, a second study using just two species, the acari-bodo and the jaraqui, focused on the influence of oxygenation status on pH_e/pH_i relationships and their adrenergic responsiveness. Red blood cell suspensions were prepared as outlined above, split into two pools, and equilibrated with either humidified O_2 or

humidified N_2 for 2 h; measured P_{O_2} values (Radiometer E5046 electrode) values were >73 kDa (550 Torr) and <0·1 kDa (1 Torr). Paired samples were then subjected to the control and adrenaline treatments under oxygenated and deoxygenated conditions respectively. Only pH_e and RBC pH_i were measured.

CALCULATIONS

The pH gradient across the RBC membrane was calculated as $pH_e - pH_i$. Two separate indices of possible RBC swelling were calculated, the Hb/Ht ratio or mean corpuscular haemoglobin concentration (MCHC) in g Hb ml RBC^{-1}, and the Ht/RBC count ratio or mean corpuscular volume (MCV) in μm^3. The amount of Na^+ taken up per ml of RBCs was calculated from the decrease in total extracellular Na^+ content (ΔNa^+) between paired control and adrenaline-treated samples. The pre-adrenaline RBC volume was used in this calculation. The levels of all organic phosphates have been normalized to the Hb concentration (i.e. µmol nucleoside phosphate per µmol Hb, assuming a Hb molecular weight of 67 kD) to avoid the effect of possible changes in RBC volume. Data have been expressed as means ± 1 S.E. (*n*), where *n* refers to the number of different fish tested. In view of the paired design, all statistical evaluations employed Student's two-tailed paired *t*-test at $P<0·05$.

RESULTS

The results were internally consistent amongst four potential measures of RBC adrenergic responsiveness. Thus adrenaline (10^{-4} M) caused a significant decrease in the pH_e–pH_i gradient across the RBC membrane [Fig. 1(a)] and a correlated significant uptake of Na^+ [Fig. 1(b)] in both tambaqui and jaraqui. In addition, the RBCs of both species clearly swelled as evidenced by a significant increase in MCV [Fig. 2(a)] and a significant decrease in MCHC [Fig. 2(b)]. For all four indices, the effects were slightly larger in tambaqui than in jaraqui. In contrast, piranha, aracu, piranambu, and acari-bodo exhibited no significant changes in the pH_e–pH_i gradient [Fig. 1(a)], no significant uptake of Na^+ [Fig. 1(b)], and no significant changes in MCV [Fig. 2(a)] or MCHC [Fig. 2(b)] in response to adrenaline.

Erythrocytic organic phosphates (per unit Hb) were completely unresponsive to adrenaline in these fish (Fig. 3), even in the two species (tambaqui, jaraqui) showing clear evidence of Na^+/H^+ exchange activation. For all species, there were no significant changes in any of the adenylates, any of the guanylates, their totals, or the total NTP (i.e. GTP + ATP).

There were pronounced differences amongst species in many of the measured parameters, but only RBC size seemed to correlate with the presence or absence of adrenergic responsiveness. Individual RBCs were smallest in the two responsive species (118 μm^3 in tambaqui and 135 μm^3 in jaraqui) and ranged from 153 μm^3 (piranambu) to a maximum of 225 μm^3 (acari-bodo) in the non-responding species [Fig. 2(a)]. On the other hand, jaraqui and tambaqui exhibited Hb/Ht ratios (i.e. MCHC) in the midrange ($\sim$0·25 g Hb ml^{-1}) between lower values in piranha, aracu, and acari-bodo ($\sim$0·22 g Hb ml^{-1}) and a much higher value in piranambu (0·31 g Hb ml^{-1}). The transmembrane pH gradient [Fig. 1(a)] was smallest in jaraqui and aracu (0·21–0·25 units), intermediate in acari-bodo and tambaqui (0·28–0·31 units) and largest in piranambu and piranha (0·37–0·42 units). The most striking differences amongst species

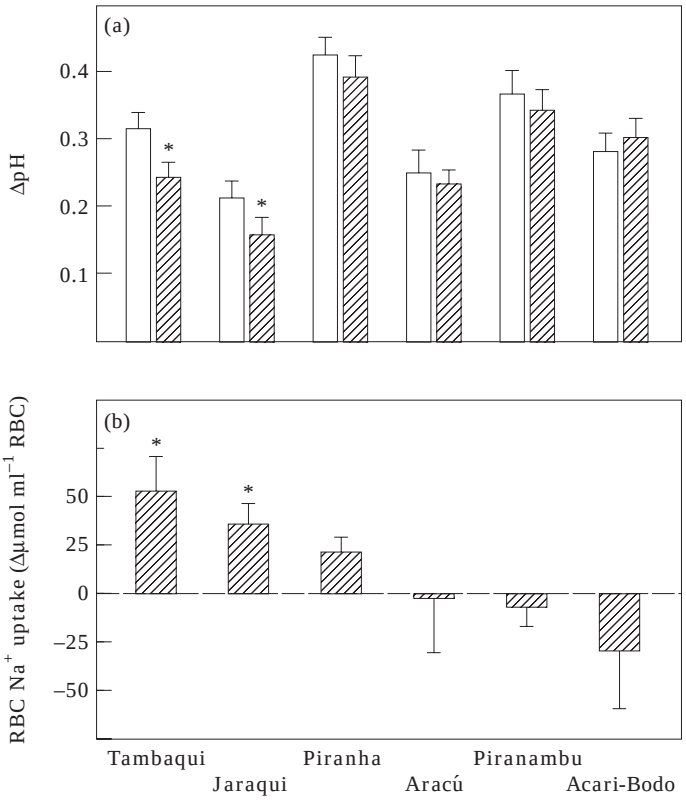

FIG. 1. (a) Changes in the transmembrane pH gradient across red blood cells (RBC pH_e–pH_i) and (b) Na^+ uptake by RBCs after stimulation by 10^{-4} M L-adrenaline in six different Amazonian teleosts: tambaqui (n=8), jaraqui (n=9), black piranha (n=6), aracu (n=7), piranambu (n=4), and acari-bodo (n=6). Means ± 1 S.E. Asterisks indicate significant differences (P<0·05) between paired control and adrenaline-treated samples in (a), and net Na^+ uptake in adrenaline-treated samples significantly different from 0 in (b). In (b), positive values represent a net removal of Na^+ from the extracellular medium. □, Control; ▨, adrenaline.

were in erythrocytic phosphates, both in relative and absolute levels (Fig. 3). In all species, AMP, ADP, GMP, and GDP levels were low relative to their respective triphosphates, and for all GTP dominated over ATP. However, the GTP : ATP ratio varied from 6 : 1 in tambaqui to 1·5 : 1 in jaraqui and aracu, whereas the absolute levels of NTP (ATP + GTP) were highest in tambaqui ($\sim$1·5 μmol μmol Hb^{-1}) and lowest in jaraqui and aracu (0·7–0·8 μmol μmol Hb^{-1}).

In some teleosts which lack a clear adrenergic response under normoxic conditions, the response can be revealed under anoxic conditions; in others, the response under normoxia is potentiated under anoxia (see Discussion). However, in acari-bodo, the lack of an adrenergic response persisted under anoxia, whereas in jaraqui, there was a slight (not significant) potentiation of the response (Fig. 4). Notably, deoxygenation caused a large increase ($\sim$0·20 units) in both pH_e and pH_i in jaraqui, but had no effect on either parameter in acari-bodo.

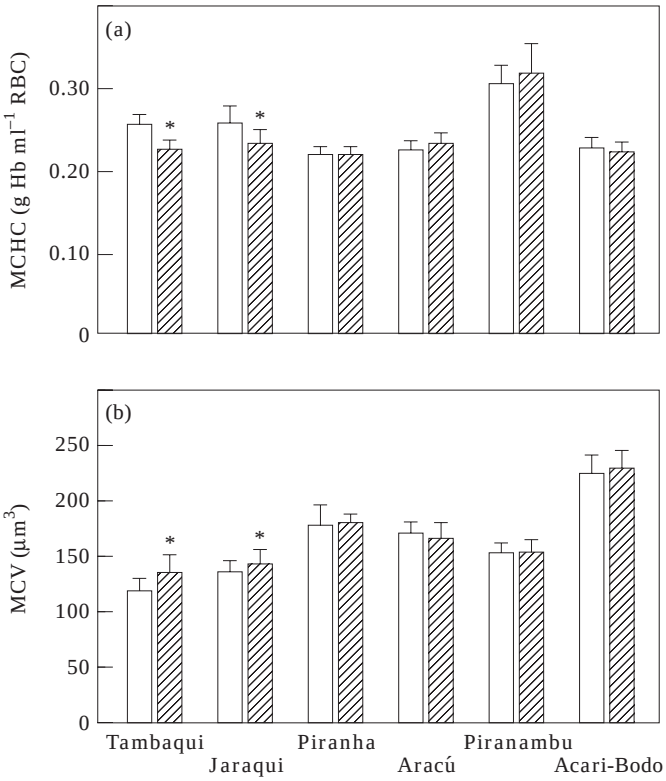

FIG. 2. Changes in (a) the mean erythrocytic corpuscular volume (MCV), calculated as the haematocrit to RBC count ratio, and (b) the mean erythrocytic corpuscular haemoglobin concentration (MCHC), calculated as the haemoglobin to haematocrit ratio, after stimulation by 10^{-4} M L-adrenaline in six different Amazonian teleosts. Other details as in Fig. 1.

DISCUSSION

The two orders examined in the present study are by far the largest groups of teleosts in the Amazon; the Characiformes comprise 45% of all species present, and the Siluriformes an additional 37% (Val & Almeida-Val, 1995). Our results show clearly that adrenergic Na^+/H^+ exchange is present in the RBCs of two members of the Characiformes, the tambaqui and the jaraqui, and apparently absent in two members of the Siluriformes, the piranambu, and the acari-bodo. This brings to six the number of teleost orders (Characiformes, Salmoniformes, Pleuronectiformes, Gadiformes, Cypriniformes, Perciformes) in which the presence of the mechanism has been seen, and two (Anguilliformes, Siluriformes) in which all evidence at present is negative (see Introduction). The silurid catfish are rather sluggish bottom-feeders while the characids in general, and the tambaqui and jaraqui in particular, are wide-ranging, highly active fish with an excellent tolerance for both hypoxia (Val & Almeida-Val, 1995) and low pH (Gonzalez *et al.*, 1998). It is therefore not surprising that the mechanism is present in the tambaqui and jaraqui. Furthermore, the fact that these two species had the smallest erythrocytes may be significant from the viewpoint

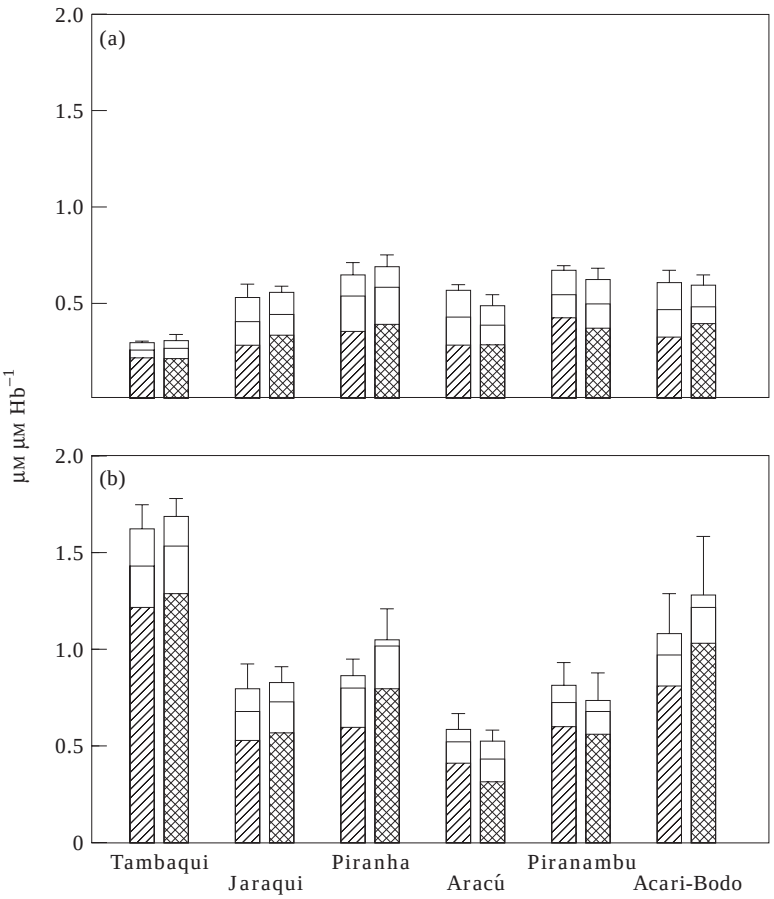

FIG. 3. (a) Adenosine phosphates (AMP, ADP and ATP; top, centre and bottom of bars, respectively) and (b) guanosine phosphates (GMP, GDP and GTP; top, centre and bottom of bars, respectively) in the RBCs of six different Amazonian teleosts before (control) and after stimulation by 10^{-4} M L-adrenaline. Values were normalized to haemoglobin to remove effects of changes in RBC volume. There were no significant differences between paired control and adrenaline-treated samples ($P>0{\cdot}05$). ▨, Control; ▩, adrenaline. Other details as in Fig. 1.

of surface area-to-volume considerations. A given cell surface density of transporters would have the greatest effect in these small RBCs.

However, it is surprising that there was no evidence for this mechanism in two of the other characids with similarly active lifestyles—the black piranha and the aracu, especially since the tambaqui and the piranha are both members of the same family Serrasalmidae. Certainly, there is precedence for very different adrenergic responsiveness in related species, e.g. the unresponsive starry flounder (Milligan & Wood, 1987) *v.* the highly responsive European flounder (Thoroed *et al.*, 1995). However, it is more difficult to show that a response is truly absent than truly present. In this regard, it must be remembered that our experiments were performed in the field, necessitating the use of a HEPES-buffered rather than HCO_3^--buffered extracellular medium. Nevertheless, similar media have been used to demonstrate the presence of the mechanism in other teleost species

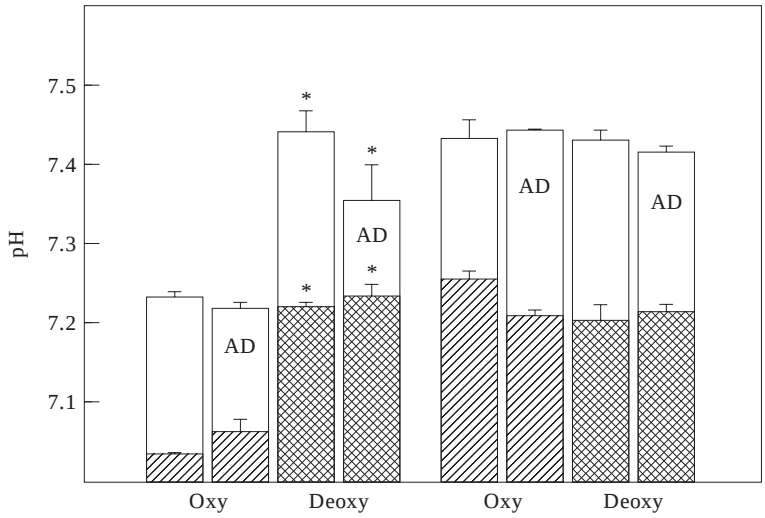

FIG. 4. RBC intracellular pH_i and extracellular pH_e in the RBCs of jaraqui (left) before (control) and after stimulation by 10^{-4} M L-adrenaline (AD) under either fully oxygenated conditions (equilibrated with O_2) or fully deoxygenated conditions (equilibrated with N_2). Means ± 1 S.E. (n=3 throughout). Asterisks indicate significant differences (P<0·05) between paired oxygenated and deoxygenated samples within each species. The decrease in pH_e–pH_i caused by adrenaline was significant in jaraqui under both oxygenated and deoxygenated conditions, but did not occur in acari-bodo (right) under either condition. pH_e, Top of bars; pH_i, bottom of bars.

(Nikinmaa & Huestis, 1984; Thoroed *et al*., 1995; Gallardo Romero *et al*., 1996). Furthermore, a mildly acidic extracellular pH_e (7·4) was set, as this is widely reported to potentiate adrenergic responsiveness (Nikinmaa & Tufts, 1989; Nikinmaa, 1992), and extensive washing and overnight storage of the RBCs was employed to overcome any desensitization associated with endogenous catecholamine mobilization during sampling (Bourne & Cossins, 1982; Walsh *et al*., 1998). A conservative conclusion would be that the adrenergic Na^+/H^+ exchange mechanism is not apparent in these species under conditions where it is readily apparent in other species, including two of the present study.

In many species, adrenergic responsiveness is potentiated by hypoxic or anoxic incubation of the blood (reviewed by Motais *et al*., 1992; Nikinmaa, 1992), and in some such as carp (Salama & Nikinmaa, 1988) the response can be seen only if the samples are subjected to greatly reduced P_{O_2} (and/or acidified to a pH_e below 7·5). For this reason, the anoxia/hyperoxia comparisons were performed on jaraqui and acari-bodo. The lack of an adrenergic response even under completely deoxygenated conditions reinforces its true absence in acari-bodo. The fact that both pH_i and pH_e were clearly elevated by deoxygenation in jaraqui but not in acari-bodo is strong evidence for the presence of a marked Haldane effect in the former and its functional absence in the latter (Wood *et al*., 1994). Previous studies indicate that in fact acari-bodo possesses a small Haldane effect (maximum number of protons released from Hb per mole of oxygenated/deoxygenated tetramer at pH 7·0, ΔZ_{Hmax}=0·44) relative to rainbow trout (ΔZ_{Hmax}=2·6) (Brauner & Val, 1996). As the Haldane effect is linked functionally to the Root effect, these data support further the argument that the presence

of an adrenergic Na^+/H^+ exchange mechanism on the RBCs is to offset the reduction in blood oxygen carrying capacity caused via the Root effect during extracellular acidification (Salama & Nikinmaa, 1988; Tufts & Randall, 1989; Cossins & Kilbey, 1991).

In terms of changes in pH_e–pH_i gradient, MCHC, MCV, and Na^+ uptake, the adrenergic responses in tambaqui and jaraqui appear to be similar to other species examined (Motais *et al*., 1992; Nikinmaa, 1992). However, the one major difference is the complete absence of any changes in erythrocytic nucleoside phosphates in response to adrenaline in both of these Amazonian fish. Most studies on salmonids (Boutilier & Ferguson, 1989; Nikinmaa, 1992; Walsh *et al*., 1997) as well as several other species (Nikinmaa *et al*., 1984; Salama & Nikinmaa, 1988) have reported a modest drop (15%) in intracellular NTP levels (normalized to Hb) in adrenergically stimulated RBCs under normoxic conditions. Under hypoxic conditions, the adrenergically stimulated fall in NTP is much greater. ATP rather than GTP appears to be the moiety which decreases. The fall under aerobic conditions is thought to represent a resetting of intracellular ATP levels subsequent to activation of Na^+,K^+ATPase (see Introduction). Perhaps the erythrocytes of these Amazonian fish simply defend an unaltered intracellular NTP level by activating increased NTP production. In addition, both tambaqui and jaraqui have a large pool of GTP ($\sim$6 : 1 and $\sim$1·7 : 1, GTP : ATP, respectively) relative to salmonids (1 : 9·5) which, in addition to the normally high levels of Fe-GTP detected in these fish species (Val & Almeida-Val, 1995), could be converted to ATP via inosine monophosphate. Increased NTP production and increased conversion of NTP complexes (e.g. Fe-GTP, Mg-ATP, and Mg-GTP) to ATP would replace the ATP at a rate which keeps pace with its utilization during adrenergic stimulation of red blood cells in these Amazonian fish species.

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