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Responses of an Amazonian Teleost, the Tambaqui (*Colossoma macropomum*), to Low pH in Extremely Soft Water

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ABSTRACT

Our goal was to compare the internal physiological responses to acid challenge in an acidophilic tropical teleost endemic to dilute low-pH waters with those in nonacidophilic temperate species such as salmonids, which have been the subject of most previous investigations. The Amazonian tambaqui (*Colossoma macropomum*), which migrates between circumneutral water and dilute acidic "blackwater" of the Rio Negro, was exposed to a graded low-pH and recovery regime in representative soft water ($\text{Na}^+ = 15$, $\text{Cl}^- = 16$, $\text{Ca}^{2+} = 20 \mu\text{mol L}^{-1}$). Fish were fitted with arterial catheters for repetitive blood sampling. Water pH was altered from 6.5 (control) to 5.0, 4.0, 3.0, and back to 6.5 (recovery) on successive days. Some deaths occurred at pH 3.0. Throughout the regime, there were no disturbances of blood gases (O_2 and CO_2 tensions and contents) or lactate

levels, and only very minor changes in acid-base status of plasma and red cells. However, erythrocytic guanylate and adenylyate levels increased at pH's less than or equal to 5.0. Down to pH 4.0, plasma glucose, cortisol, and total ammonia levels remained constant, but all increased at pH 3.0, denoting a stress response. Plasma Na^+ and Cl^- levels declined and plasma protein concentration increased at pH 3.0, indicative of ionoregulatory and fluid volume disturbance, and neither recovered upon return to pH 6.5. Cortisol and ammonia elevations also persisted. Transepithelial potential changed progressively from highly negative values (inside) at pH 6.5 to highly positive values at pH 3.0; these alterations were fully reversible. Experimental elevations in water calcium levels drove the transepithelial potential positive at circumneutral pH, attenuated or prevented changes in transepithelial potential at low pH, and reduced Na^+ and Cl^- loss rates to the water during acute low-pH challenges. In general, tambaqui exhibited responses to low pH that were qualitatively similar but quantitatively more resistant than those previously documented in salmonids.

Introduction

Our present understanding of the physiological responses of freshwater fish to low environmental pH has resulted largely from studies stimulated by the acid rain problem, on species such as salmonids, which do not naturally occur in waters of low pH (reviewed by Fromm [1980]; Wood and McDonald [1982]; McDonald [1983]; Potts and McWilliams [1989]; Wood [1989]; Reid [1995]). Physiological responses to acid stress have also been studied in other nonacidophilic groups such as cyprinids (Ultsch et al. 1981; van Dijk et al. 1993) and cichlids (Wendelaar Bonga et al. 1987). At extremely low pH's (e.g., 2.5–4.0), death results from a rapid suffocation response due to a breakdown of gill structure and massive branchial mucus production. However, at more environmentally realistic pH's (e.g., 4.0–5.5), key toxic responses of such nonacidophilic species all relate to a disturbance of ionoregulation by high external H^+ levels. Inhibition of active Na^+ and Cl^- uptake at the gills, combined with stimulation of passive ion effluxes, causes decreases in plasma Na^+ , Cl^- , and osmolality levels and an associated fluid shift from extra- to intracellular compartments. This results in decreased blood volume, increased hematocrit (hct), and increased plasma protein concentrations. The combination of high blood viscosity and low blood volume may eventually kill the fish through circulatory failure (Milligan and Wood 1982). If, however, the fish are able to adjust to

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lowered pH during continued exposure, they do so mainly by controlling the branchial efflux component rather than by restoring influx (Wood 1989).

Higher water calcium levels ($[Ca^{2+}]$) offer considerable protection against these damaging effects, apparently by Ca^{2+} versus H^+ competition for sites on tight junctions that maintain branchial epithelial integrity, thereby reducing effluxes (McDonald 1983). Higher $[Ca^{2+}]$ also minimizes the large changes in branchial transepithelial potential seen at low pH (Potts and McWilliams 1989). Curiously, there is little disturbance of acid-base balance at low pH when external $[Ca^{2+}]$ is low, but higher water $[Ca^{2+}]$ promotes the uptake of acidic equivalents and the development of internal acidosis (McDonald et al. 1980; Wood 1989).

While the occurrence of teleosts in naturally acidified waters has long been known (see, e.g., Jewell and Brown 1924; Brown and Jewell 1926), only a very few physiological studies have been performed on acidophilic species naturally occurring in waters of low pH (i.e., <5.5 ; reviewed by Gonzalez [1996]). These have been species such as sunfish, perch, and mudminnows endemic to acid bogs in the Northern Hemisphere (Dederen et al. 1986; Gonzalez and Dunson 1987, 1989a, 1989b; Freda and McDonald 1988; McDonald et al. 1991). In general, the results have been qualitatively similar to but quantitatively different from those of species that normally live at circumneutral pH. Smaller ionic disturbances occur at lower pH thresholds in the acidophilic species. The role of Ca^{2+} is unclear. In sunfish, the gills have a very high Ca^{2+} -binding affinity, such that differences in the protective effects of $[Ca^{2+}]$ are seen only at a very low range of external $[Ca^{2+}]$ (<100 – $125 \mu\text{mol L}^{-1}$; Gonzalez and Dunson 1987, 1989a), whereas in perch, the gill appears to be insensitive to changes in water $[Ca^{2+}]$, at least above $35 \mu\text{mol L}^{-1}$, the lowest level tested (Freda and McDonald 1988). Effects of low pH and $[Ca^{2+}]$ on transepithelial potential and acid-base balance have not been examined in acidophilic species, and it is not known whether gas exchange is adversely affected at extremely low pH's in such fish.

The Amazon basin is home to more than 20% of the world's freshwater ichthyofauna, a significant portion of which (about 40%) inhabits the "blackwaters" of the Rio Negro and its tributaries (Val and Almeida-Val 1995). Here, fish encounter some of the most dilute, naturally acidic waters seen anywhere on earth, reflecting the unique geochemistry of the headwaters and the input of humic and fulvic acids from the breakdown of jungle vegetation. While the pH of the Rio Negro itself is around 5.5, with Na^+ , Cl^- , and Ca^{2+} levels generally less than $50 \mu\text{mol L}^{-1}$, forest streams routinely exhibit pH's as much as 2 pH units lower and ion levels only 20% of those in the main river (Furch 1984; Val and Almeida-Val 1995; Walker and Henderson 1996). Many species enter these forest streams to feed or even reproduce, and many naturally migrate between circumneutral and low-pH environments (Val and Almeida-Val 1995).

The resistance of certain native Amazon species to low pH

has long been recognized (Dunson et al. 1977), but only very recently have the mechanisms involved been investigated. Gonzalez et al. (1997) found the surprising result that branchial efflux is relatively sensitive and influx is resistant to low pH in the blackskirt tetra; elevated $[Ca^{2+}]$ is protective against Na^+ loss at low pH, but the gills exhibit a low affinity for Ca^{2+} . In a study on several species collected freshly from the Rio Negro, Gonzalez et al. (1998) reported only modest net losses of Na^+ and Cl^- down to a pH of 3.0–3.5, with negligible protective effects of Ca^{2+} . To date there has been no information on internal responses.

The present study examined the internal responses (blood gases, plasma ions, acid-base status, protein, metabolites, cortisol, transepithelial potential, and red blood cell physiology) of the Amazonian tambaqui (*Colossoma macropomum*) to a graded low-pH regime. In addition, the importance of external $[Ca^{2+}]$ in affecting ion flux rates and changes in transepithelial potential at low pH was evaluated. The tambaqui was selected because it is reported to migrate from circumneutral water to acidic blackwater of the Rio Negro and its tributaries to feed on forest fruits during the rainy season (Goulding 1980; Goulding and Carvalho 1982; Roubach and Saint-Paul 1994; Val and Almeida-Val 1995). *C. macropomum* is a member of the most common order in the Amazon basin, the Characiformes, and is now a species of great economic importance (Goulding and Carvalho 1982; Val and Honczaryk 1995). A particular goal was to compare the responses of the tambaqui with earlier data on nonacidophilic fish such as salmonids.

Material and Methods

Experimental Animals

Tambaqui (99–1,640 g, $N = 48$) were obtained from commercial aquaculture (the Amazon Fish Farm near Itacoatiara City, about 200 km from Manaus) in November 1995. There the fish had been raised at a density of one adult fish per square meter in large, shallow outdoor ponds at 25° – 37°C with O_2 saturation maintained close to 100%. The composition of the water flushing the ponds varied temporally from circumneutral "whitewater" (pH 7.5), at some times, to acidic groundwater (pH 4.0) more representative of dilute blackwater at other times. Thus the fish had experienced a range of water acidities and ionic levels before the present experiments.

In our laboratory, the fish were held without feeding for 7 d before experiments in 800-L tanks. The tanks were filled with groundwater obtained from a well on the campus of the National Institute for Amazon Research, Manaus. Before placing fish in the groundwater, it was necessary to vigorously aerate it so as to remove high levels of dissolved CO_2 , thereby raising pH from 4.1 to 6.0–6.5. In addition, the basal $[Ca^{2+}]$ ($9 \mu\text{mol L}^{-1}$) was raised to $20 \mu\text{mol L}^{-1}$ by addition of $Ca(NO_3)_2$. Measured water composition was (in $\mu\text{mol L}^{-1}$): Na^+ , 15; Cl^- , 16; K^+ , 9; Ca^{2+} , 20; Mg^{2+} , 2; NO_3^- , 37; SO_4^{2-} , 5; total phosphate,

1. Dissolved organic carbon concentration was 2.05 mg L^{-1} , pH was about 6.5, and temperature was $28^{\circ}\text{--}30^{\circ}\text{C}$. This very dilute water, close to natural blackwater apart from its lower organic content, was used in all experiments.

In preparation for all experiments except series 3 (see below), the fish were anaesthetized with MS-222 (0.5 g L^{-1} , neutralized to pH = 6.0 with KOH), placed on an operating table, and fitted with an indwelling catheter for repetitive blood sampling and/or transepithelial potential measurements. The very small size of the mouth precluded the traditional dorsal aortic catheterization technique, so instead the caudal artery (or in a few cases the caudal vein) was cannulated in the tail region. This involved surgically separating the hypaxial and epaxial muscle masses so as to expose the hemal arch, and then inserting the catheter under a vertebral spine and advancing it several centimeters into the vessel. PE50 tubing (Clay-Adams, Parsippany, N.J.) was used, filled with Cortland saline (Wolf 1963) heparinized at 50 i.u. mL^{-1} with lithium heparin (Sigma, St. Louis). The wound was dusted with oxytetracycline (Sigma) and tightly closed with silk sutures.

The fish were allowed to recover for 36 h in their experimental chambers, separate light-shielded polyethylene containers (volume of 8 L in series 1, 3.5 L in series 2) served with individual aeration and a flow of 1.5 L min^{-1} of recirculated water. The fish chambers were housed on a wet table that drained back into the recirculation reservoir. Total volume of the system, serving a maximum of 12 fish, was 760 L; 90% of the water was replaced twice a day.

Experimental Series

Series 1. This series used the largest fish available (490–1,640 g; $N = 12$). Repetitive blood sampling was used to monitor the internal responses of cannulated tambaqui to a graded acid exposure; H_2SO_4 was used to lower water pH. On each sampling day at about 1000 hours, after an overnight period at a given pH, the water in the reservoir was replaced with new water of the same pH, without interruption of flow to the fish chambers. Blood and water samples were taken and transepithelial potentials were measured between 1400 and 2000 hours on successive days at pH's of 6.5 (control), 5.0, 4.0, 3.0, and 6.5 again (recovery). On each day, the water was changed again at about 2000 hours and gradually adjusted to the new pH over the next 4 h. Thus fish had experienced each pH for about 17 h at the time of each sampling.

At each sample time, a water sample was drawn from in front of the fish's mouth for measurement of water O_2 tension (P_{wO_2}). Transepithelial potential was measured via the catheter, and then blood samples (1,000 μL) were drawn into two gastight, ice-cold 500- μL Hamilton syringes and immediately apportioned for analyses. Approximately 200 μL of blood (recovered from electrodes) plus 800 μL of Cortland saline were reinfused into the fish after sampling to restore blood volume. Plasma was separated by centrifugation of an aliquot at 10,000

g for 2 min, and the red blood cell (RBC) pellet was immediately frozen in liquid N_2 for measurement of intracellular pH (RBC pHi). Extracellular pH (pHe) that was usually the pH of arterial blood (pHa), RBC pHi, the partial pressure of O_2 in arterial blood (PaO_2), the total concentration of O_2 in arterial blood (CaO_2), the total concentration of CO_2 in arterial plasma (CaCO_2), hematocrit (hct), and hemoglobin concentration (Hb) were measured immediately. Plasma Na^+ , K^+ , Ca^{2+} , Cl^- , protein, total ammonia, glucose, and cortisol were measured after storage at -70°C . Blood samples (100 μL) for determination of lactate and RBC nucleoside phosphates (ATP, ADP, AMP, GTP, GDP, and GMP) were immediately deproteinized in 200 μL ice-cold 8% perchloric acid for several hours. The slurry was spun briefly, and then the supernatant was decanted, neutralized with 6 mol L^{-1} KOH, and then stored at -70°C for later analysis.

Series 2. This series used fish of intermediate size (190–440 g; $N = 18$) to examine the acute responses of transepithelial potential to alterations in water [Ca^{2+}] and pH. Cannulated tambaqui were placed overnight in small containers of known volume (generally 3.5 L) with individual flow and aeration. In one experiment, the flow was stopped and the water changed to groundwater that had purposely not been supplemented with $\text{Ca}(\text{NO}_3)_2$ in order to start the series at a very low [Ca^{2+}] ($9 \mu\text{mol L}^{-1}$). After a 10-min settling period, transepithelial potential was measured, and then known volumes of a concentrated stock solution of $\text{Ca}(\text{NO}_3)_2$ were added at 15-min intervals so as to sequentially raise the water [Ca^{2+}] from approximately 10 to approximately 10,000 $\mu\text{mol L}^{-1}$ in half-log-unit steps. The pH was maintained at about 6.5. At each step, transepithelial potential was measured via the catheter after 10 min, and a water sample was taken at this time in order to determine the exact [Ca^{2+}]. In a second experiment with a similar protocol, water Ca^{2+} was set to approximately 20, 200, or 10,000 $\mu\text{mol L}^{-1}$ by $\text{Ca}(\text{NO}_3)_2$ addition at pH 6.5, and then pH was sequentially lowered to pH 3.0 by addition of H_2SO_4 at 15-min intervals.

Series 3. This series used small tambaqui (99–288 g; $N = 24$) to examine the effect of acute exposure to pH 3.5 on net whole-body Na^+ and Cl^- flux rates at different water [Ca^{2+}] values. Noncannulated fish were allowed to settle for several hours in one of six 3.5-L chambers served with individual aeration and recirculation flow from a 200-L reservoir of water with a [Ca^{2+}] of $20 \mu\text{mol L}^{-1}$, pH = 6.5. The flow was then stopped for 60 min, during which the individual chambers were operated as closed systems to make control flux measurements. Water samples (20 mL) were taken at the beginning and end of the hour for analysis of [Na^+] and [Cl^-]. In the reservoir, water pH was then lowered to pH 3.5 with H_2SO_4 , and water [Ca^{2+}] was either kept at $20 \mu\text{mol L}^{-1}$ or raised to 100 or 700 $\mu\text{mol L}^{-1}$ with $\text{Ca}(\text{NO}_3)_2$ in different experiments. The chambers were flushed for 10 min with this low-pH water from the reservoir

and then closed again for another 60-min flux measurement. Measurements of pH in each chamber at the end of the experimental flux showed that pH rose by only 0.05 units on average. A parallel experiment was also performed at the lowest water $[Ca^{2+}]$, in which a Ca^{2+} -antagonist, 20 $\mu\text{mol L}^{-1}$ lanthanum chloride ($LaCl_3$), rather than low pH, was used as the challenge during the second flux period.

Analytical Techniques

All gas and pH measurements were made with electrodes (Radiometer, Copenhagen) connected to Radiometer pHM 71, 72, or 84 acid-base analyzers. Whole-blood CaO_2 was measured by the method of Tucker (1967); plasma $CaCO_2$ was measured by the method of Cameron (1971); and RBC pHi was measured by the freeze-thaw method of Zeidler and Kim (1977). Water pH was monitored with a GK2401C glass combination electrode; whole-blood pHe and RBC pHi were determined with an E5021a "gun" micro-electrode system; and PwO_2 and PaO_2 were determined with an E5036 electrode. The Tucker and Cameron chambers were fitted with E5036 and E5046 electrodes, respectively. All electrodes were thermostatted to the experimental temperature, except those in the Cameron and Tucker chambers, which were operated at 40°C.

Hct was determined by centrifugation at 5,000 g for 5 min, and Hb was measured by the cyanmethemoglobin method (Sigma reagents). Plasma and water $[Na^+]$, $[Ca^{2+}]$, and $[K^+]$ were determined with a CELM model FC108 flame photometer (CELM, Rio de Janeiro), and $[Cl^-]$ was measured by the colorimetric assay of Zall et al. (1956). Plasma total ammonia, glucose, and whole-blood lactate concentrations were determined enzymatically using Sigma kits (nos. 171, 16, and 826, respectively). Cortisol was measured by an $[^{125}I]$ radioimmunoassay (ICN Immunocorp, Montreal) using standards diluted to the protein concentrations found in tambaqui plasma and analyzed on a Canberra-Packard Minaxi 5000 gamma counter (Downers Grove, Ill.).

RBC adenylates (ATP, ADP, and AMP) and guanylates (GTP, GDP, and GMP) were measured by high-pressure liquid chromatography (HPLC) using an LKB 2152 HPLC controller and 2150 titanium pump coupled to a 2220 recording integrator (LKB, Turku, Finland). The separation was performed on an Aquapore (PMI Products, Ithaca, N.Y.) AX-300 7- μm weak anion exchanger eluting at 2 mL min^{-1} according to the methods of Val et al. (1994).

Transepithelial potential was determined by means of 3-mol L^{-1} KCl-agar bridges connected via Ag/AgCl electrodes to a high-impedance voltmeter (Perry and Wood 1985). The reference electrode was placed in the water in the fish chamber, and the measurement electrode was connected to the blood via the catheter. The system was calibrated to zero potential by placing both electrode KCl-agar tips in the water.

Calculations, Statistics, and Display of Data

Arterial CO_2 tension ($PaCO_2$) and plasma $[HCO_3^-]$ were calculated from measured $CaCO_2$ and pHa values (i.e., pHe) via the Henderson-Hasselbalch equation using appropriate solubility coefficients and pK^1 values from Boutilier et al. (1984). Mean cell Hb concentration was calculated as the ratio of Hb (g mL^{-1}) to hct (mL RBC mL^{-1}). The levels of all RBC nucleoside phosphates were normalized to the Hb concentration (i.e., micromoles of nucleoside phosphate per micromole of Hb, assuming a Hb molecular weight of 67,000 D) to avoid the effects of possible changes in RBC volume. O_2 saturation of the Hb was calculated by subtracting the physically dissolved component (the product of PaO_2 and the O_2 solubility coefficient; from Boutilier et al. [1984]) from the total O_2 content (CaO_2 , mmol L^{-1}) and then dividing by 4 times $[Hb]$ (mmol L^{-1} ; i.e., assuming four O_2 -binding sites per Hb molecule). Flux rates of Na^+ and Cl^- were calculated from measured changes in the concentration of the ion in the closed external water over the flux period, factored by the mass of the fish, chamber volume, and time.

Most data have been expressed as means \pm 1 SEM (N), where N is the number of fish. As each fish was used as its own control, all data points were compared to the original control values using Student's two-tailed paired t -test at $P \leq 0.05$, with the Bonferroni correction for multiple comparisons (Nemenyi et al. 1977). Note that in series 1, of the 12 tambaqui present at the start of the experiment, catheters failed in two animals, and another two died before measurement at the pH 3.0 step of the regime. To avoid bias from these missing data, means given in figures and tables, and their statistical assessment, were tabulated for only those fish ($N = 8$) that supplied complete data sets down to pH 3.0. Three additional fish died before the recovery measurement, so the recovery means (and their statistical assessment relative to control) are based on $N = 5$ and are joined to the rest of the data by a dashed line in the figures to indicate this change in sample size. Two of the eight fish providing data through pH 3.0 and one of the five providing data on the recovery day had caudal vein rather than caudal artery catheters. Use of the venous sampling site had no appreciable effect on most parameters, but it clearly provided different blood gas and pH values. Thus, only arterial data were used for the blood gas and pH means and statistics. Therefore, for these assessments only, $N = 6$ through pH 3.0, and $N = 4$ at recovery.

Results

Series 1: Internal Responses to a Graded Low-pH Regime

Exposure to pH's as low as 3.0 had negligible influence on blood oxygenation and transport in *Colossoma macropomum*. At an inspired PwO_2 of about 110 Torr (where 1 Torr = 133.322 Pa), tambaqui exhibited a relatively low PaO_2 of approximately

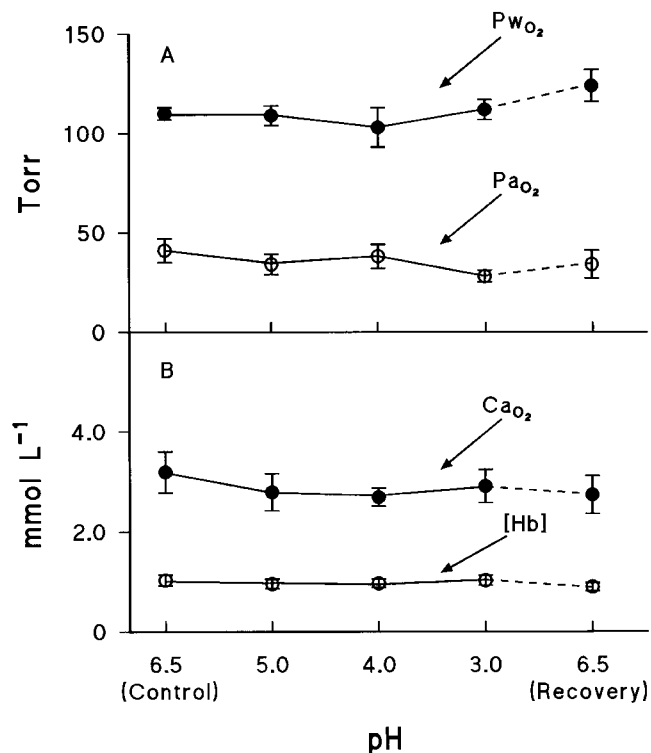


Figure 1. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on P_{aO_2} and P_{wO_2} (A) and CaO_2 and $[Hb]$ in arterial blood (B). Values are means \pm 1 SEM ($N = 6$ at control, pH 5.0, 4.0, and 3.0; $N = 4$ at recovery). There were no significant differences ($P \leq 0.05$) in any value from the respective control mean.

35 Torr, which did not change during acid exposure and recovery (Fig. 1A). Despite the progressive removal of RBCs during repeated blood sampling, Hb concentration also remained unchanged (Fig. 1B). This constancy was reflected in stable levels of CaO_2 throughout the regime (Fig. 1B). Arterial O₂ saturation ($70.7\% \pm 2.2\%$, $N = 8$ under control conditions) also remained constant (data not shown). Venous PO₂ was 1–6 Torr, providing a venous O₂ saturation of less than 30% (two fish only; data not shown).

Blood acid-base status was also barely affected by these low-pH exposures, despite the fact that at pH 3.0, external $[H^+]$ was nearly five orders of magnitude greater than that in the blood. The pH_a was about 7.80 initially and fell marginally at pH 5.0 and 4.0, but was not significantly different from the control value at pH 3.0 or at recovery (Fig. 2A). RBC pH_i, about 7.3 initially, did not change significantly down to an external pH of 4.0, but fell by about 0.1 units at pH 3.0 and did not recover when water pH was raised (Fig. 2A). P_{aCO_2} was relatively high, about 5 Torr under control conditions (Fig. 2B), where P_{wCO_2} , as estimated from water pH and titratable alkalinity measurements, was about 2 Torr. P_{aCO_2} remained stable throughout the regime. $CaCO_2$ also remained stable (data not shown), so arterial plasma $[HCO_3^-]$ ($7\text{--}9$ mmol L⁻¹) did not change significantly throughout the exposure (Fig. 2C).

Both hct and mean cell Hb concentration, an index of red blood cell size, remained unchanged at control levels ($20.9\% \pm 2.1\%$ and 0.3312 ± 0.0106 g Hb mL⁻¹, respectively, $N = 8$) throughout the exposure (data not shown). However, there were pronounced changes in RBC nucleoside phosphate levels. Guanylates clearly dominated over adenylates by about fivefold at all times, and this difference was consistent for tri-, di-, and monophosphates (Fig. 3). ATP and AMP each made up about 40% of the RBC adenylate pool, while ADP constituted the remaining 20%. GTP made up 50%–60% of the guanylate pool, with the remainder being made up of approximately equal contributions from GDP and GMP. The total erythrocytic adenylate pool (per unit Hb) increased progressively throughout the regime, almost doubling by the end. The elevation was significant at pH 4.0, pH 3.0, and at recovery. This reflected increases in both ATP (significant at all points throughout the regime) and AMP (significant at pH = 3.0 and at recovery). The total erythrocytic guanylate pool also showed a tendency to increase at low pH, although this was not significant. However, the GTP component was significantly elevated by about 40% at pH 5.0, 4.0, and recovery.

Several stress indicators demonstrated negligible effects down to pH 4.0, but a substantial disturbance at pH 3.0. Total plasma ammonia approximately doubled at pH 3.0 from control values of about $140 \mu\text{mol L}^{-1}$ and continued to rise during recovery (Fig. 4A). Both plasma glucose (initially about 4 mmol L⁻¹) and plasma cortisol concentrations (initially about $60 \mu\text{g L}^{-1}$) also approximately doubled at pH 3.0, but only glucose returned to control values at recovery (Fig. 4B, C). Blood lactate remained very low (<1 mmol L⁻¹) and did not change throughout the regime (Fig. 4B), confirming that the tambaqui encountered no O₂ delivery problem down to pH 3.0.

Considering the dilute nature of the environment, plasma $[Na^+]$, $[Cl^-]$, $[Ca^{2+}]$, and $[K^+]$ (approximately 158, 145, 8.5, and 4 mmol L⁻¹, respectively) were all relatively high under control conditions and remained unchanged at pH 5.0 and 4.0. The stress response at pH 3.0 coincided with the onset of osmoregulatory dysfunction (Fig. 5). Plasma Na^+ and Cl^- both fell significantly by about 20% relative to control values, with no evidence of restoration at the recovery measurement (Fig. 5A). Plasma protein concentration, an inverse index of changes in blood volume (McDonald et al. 1980), increased by about 20% at pH 3.0, again with no evidence of subsequent recovery (Fig. 5B). There were no significant changes in plasma $[Ca^{2+}]$ or $[K^+]$ levels throughout the exposure regime (data not shown).

Transepithelial potential between the body fluids and the external water (with the latter taken as the zero reference point) changed dramatically in response to low pH (Fig. 6). Transepithelial potential rose from a highly negative value (-23 mV) at control pH 6.5 to a highly positive value ($+35$ mV) at pH 3.0, with a complete reversal back to the control level at recovery. Crossover from negative to positive potential occurred between pH 5.0 and 4.0.

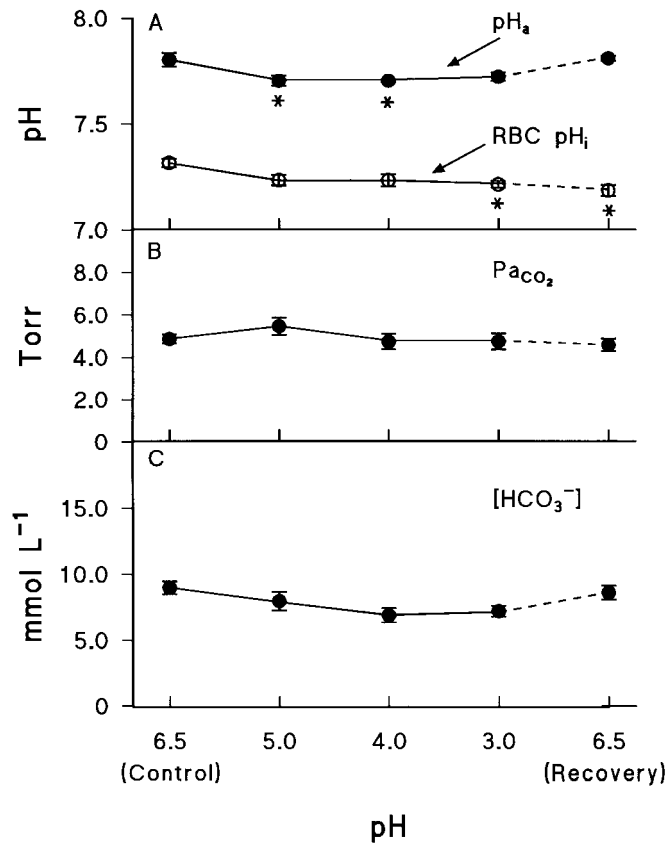


Figure 2. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on pHa (extracellular pH) and RBC pH_i of arterial blood (A), PaCO₂ in arterial blood (B), and [HCO₃⁻] in arterial blood plasma (C). Values are means \pm 1 SEM ($N = 6$ at control, pH 5.0, 4.0, and 3.0; $N = 4$ at recovery). Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean.

Series 2: Responses of Transepithelial Potential to Acute Changes in Environmental Ca²⁺ and pH

At control pH (about 6.5), transepithelial potential was sensitive to water [Ca²⁺], changing from about -30 mV at $10 \mu\text{mol L}^{-1}$ to about $+10$ mV at $10,000 \mu\text{mol L}^{-1}$ (Fig. 7). The relationship between transepithelial potential and \log [Ca²⁺] was hyperbolic, such that [Ca²⁺] changes in the low, natural range had a much greater influence than at the upper range, where an asymptote was approached.

At the control water [Ca²⁺] (about $20 \mu\text{mol L}^{-1}$), the large effects of acute reductions in water pH on transepithelial potential (Fig. 8) were very similar to those seen during the graded pH regime of series 1 (Fig. 6). The relationship was attenuated at [Ca²⁺] of approximately $200 \mu\text{mol L}^{-1}$, but the potential still increased substantially from a slightly negative value (-4 mV) at pH 6.5 to $+28$ mV at pH 3.0. However, at very high external [Ca²⁺] (about $10,000 \mu\text{mol L}^{-1}$), transepithelial potential became insensitive to water pH, remaining constant at about $+12$ mV from pH 6.5 down to 3.0 (Fig. 8).

Series 3: Responses of Net Ion Flux Rates to Acute Changes in Water Chemistry

At control pH (about 6.5) and control [Ca²⁺] (about $20 \mu\text{mol L}^{-1}$), tambaqui were in approximate ion balance in all tests, with negligible net losses of Na⁺ and Cl⁻ to the external water. However, at low pH, net ion loss rates were very sensitive to water [Ca²⁺] (Fig. 9). Acute reductions of water pH to 3.5 for 1 h caused substantial net losses of both ions, with flux rates of about $-1,500$ and $-1,000 \mu\text{mol kg}^{-1} \text{h}^{-1}$ for Na⁺ and Cl⁻, respectively, at a [Ca²⁺] of approximately $20 \mu\text{mol L}^{-1}$. These loss rates were reduced by about 50% when the experiment was performed at a [Ca²⁺] of about $100 \mu\text{mol L}^{-1}$, and by about 67% at a [Ca²⁺] of about $700 \mu\text{mol L}^{-1}$ (for Na⁺ only). When LaCl₃ rather than low pH was used as the challenge at a concentration ($20 \mu\text{mol L}^{-1}$) equimolar to water [Ca²⁺], there was no effect on ion loss rates (data not shown).

Discussion

Basic Physiology of the Tambaqui

Colossoma macropomum is now a species of immense importance for commercial fishing and aquaculture in the Amazon basin (Goulding and Carvalho 1982; Roubach and Saint-Paul 1994; Val and Honczaryk 1995), but until recently, very little

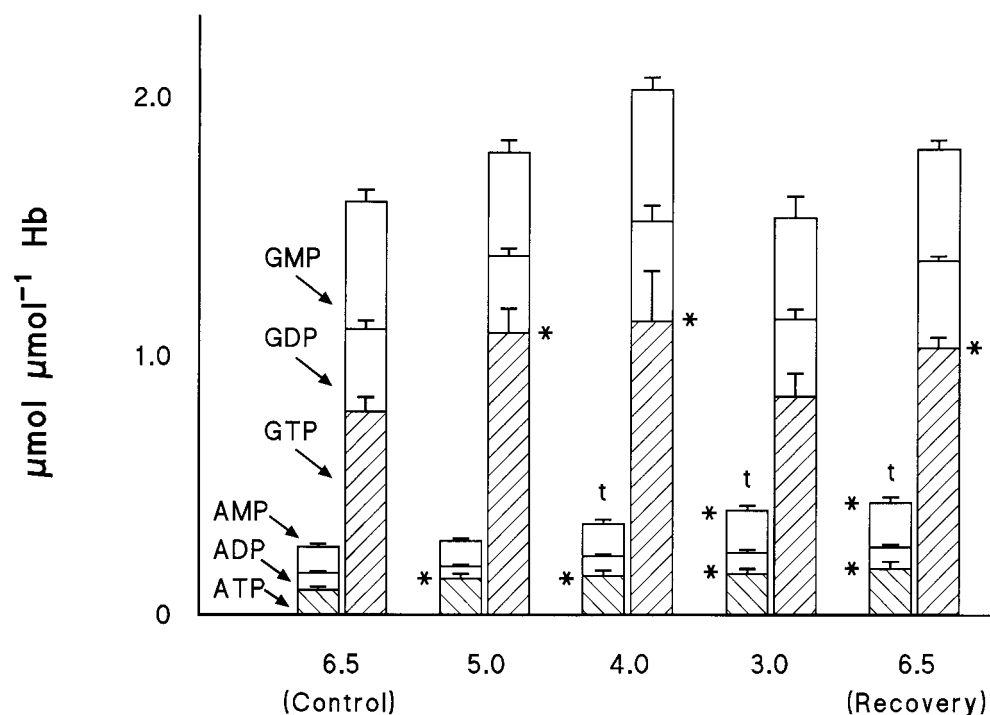


Figure 3. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on the concentrations of nucleoside phosphates in the RBCs. All concentrations are expressed in micromoles of nucleoside phosphate per micromole of Hb to correct for possible changes in RBC volume. The bar represents the sum of the three components with each fraction indicated (mean + SEM; $N = 8$ at control, pH 5.0, 4.0, and 3.0; $N = 5$ at recovery). Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean for each fraction; daggers indicate adenylate or guanylate totals significantly different ($P \leq 0.05$) from the respective control total.

has been known about its basic ionoregulatory and respiratory physiology. Most interest has centred on its exceptional tolerance to environmental hypoxia, with perfect regulation of oxygen consumption down to environmental O_2 levels of about 30% air saturation (Saint-Paul 1984). Below this point, the tambaqui exhibits a remarkable ability to “grow” a large “lip” within several hours of exposure to environmental hypoxia (Braun and Junk 1982). The tambaqui does not breathe air, but this lip mechanically facilitates “skimming” of the more O_2 -rich surface waters (“aquatic surface respiration”; Rantin and Kalinin 1996), and its appearance is accompanied by simultaneous biochemical adjustments in erythrocytes and white muscle that aid hypoxia tolerance (reviewed by Val and Almeida-Val [1995]). Recently, a pronounced Root effect (Val and Almeida-Val 1995) and a capacity for adrenergic pHi regulation in the RBCs (Val et al. 1998) have been reported. The present study augments this picture by demonstrating very low resting levels of P_{aO_2} , approximately 75 Torr below P_{wO_2} (Fig. 1A). P_{aCO_2} was correspondingly elevated in accord with the difference in solubility coefficients, being about 3 Torr above

the estimated P_{wCO_2} of 2 Torr (Fig. 2B). We hypothesize that the presence of these high partial pressure gradients between blood and water indicates a low gill diffusing capacity, despite the fact that total gill surface area is unusually large (Saint-Paul 1984). Low branchial diffusing capacity would thereby minimize ionic losses and ionoregulatory work load in dilute, acidic environments. Indeed, plasma ion levels under control conditions were high for freshwater teleosts (Fig. 5A). Most other blood parameters appeared to be within the normal range (McDonald and Milligan 1992).

The Graded Low-pH Regime

Most previous studies on the responses of fish to environmental acidity have involved large stepwise reductions in pH (e.g., 7.0 to 4.0). Arguably, such a change could represent a sudden rainstorm or snowmelt event associated with acidic precipitation, but it would have little relevance to the environmental situation in the Amazon basin. A few studies have employed a more gradual acidification regime and have concluded that in general it is better tolerated (Stuart and Morris 1985; Wendelaar Bonga et al. 1987; Van Dijk et al. 1993). Therefore, in the present study, we employed a graded regime in which the fish were exposed to progressively lower pH's on successive days. This might represent the situation of a tambaqui migrating voluntarily from circumneutral water to lower-pH blackwater and then to an extremely acidic forest stream for feeding (see, e.g., Goulding 1980; Goulding and Carvalho 1982). The recovery measurements at the end of the regime were performed to check which physiological responses were readily

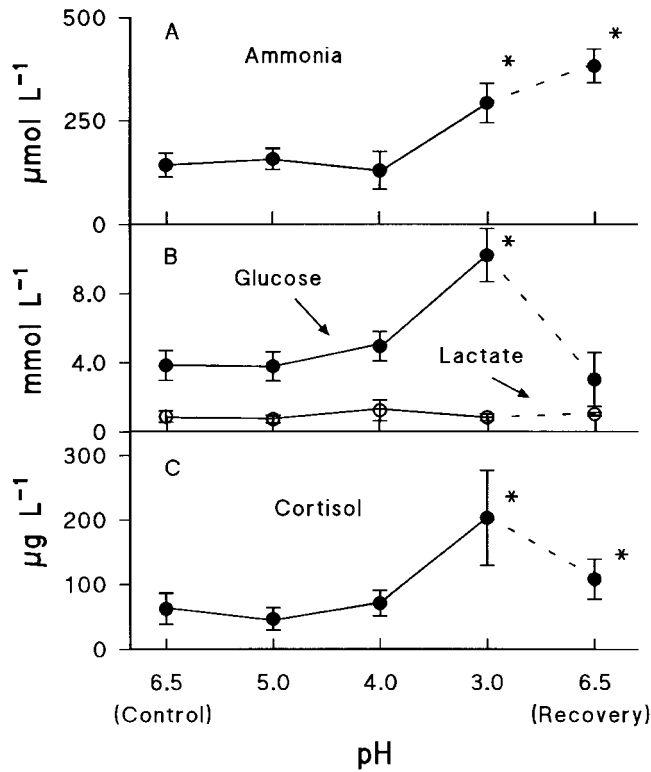


Figure 4. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on the concentration of total ammonia in blood plasma (A), the concentration of glucose in blood plasma and lactate in whole blood (B), and the concentration of cortisol in blood plasma (C). Values are means \pm 1 SEM ($N = 8$ at control, pH 5.0, 4.0, and 3.0; $N = 5$ at recovery). Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean.

reversible and which were not, with the latter being indicative of damage to the physiological mechanism(s) involved.

Internal Responses of Tambaqui to Low pH

The present study is the first to report the internal responses to low environmental pH of a member of the order Characiformes or, indeed, of any Amazonian fish that naturally inhabits blackwater. In general, the picture that emerges is qualitatively similar to but quantitatively different from that of earlier studies on nonacidophilic species of the Northern Hemisphere, such as salmonids, when these fish were exposed to low pH in soft water (reviewed by Fromm [1980]; Wood and McDonald [1982]; McDonald [1983]; Potts and McWilliams [1989]; Wood [1989]; Reid [1995]). In such species, stress responses and ionoregulatory disturbance become evident at a pH threshold of 5.0–5.5, whereas down to pH 4.0, internal changes in tambaqui were generally negligible—that is, tambaqui showed at least 10-fold greater tolerance of $[H^+]$. However, at pH 3.0, significant stress responses occurred (Fig. 4) as ionoregulatory and fluid volume disturbance developed, evidenced by declin-

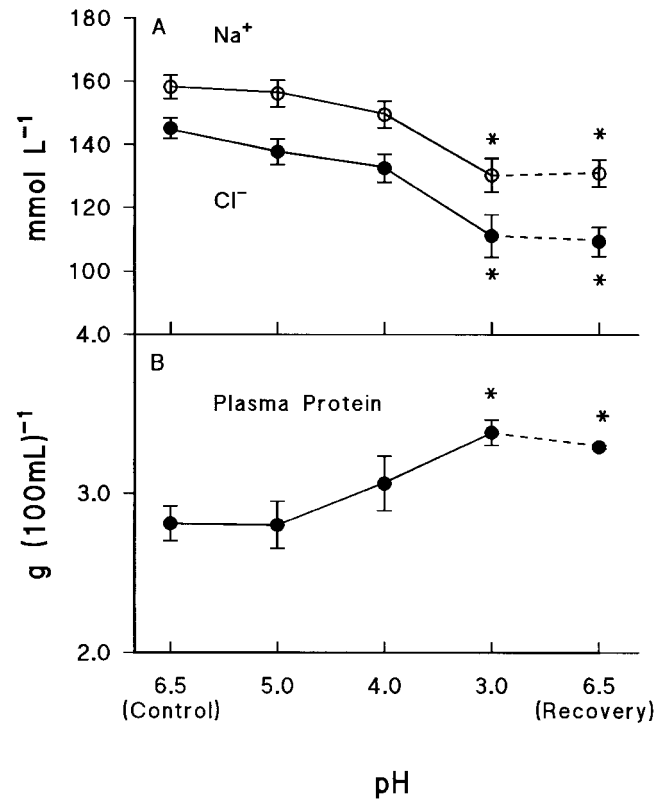


Figure 5. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on $[Na^+]$ and $[Cl^-]$ in blood plasma (A) and the concentration of plasma protein (B). Values are means \pm 1 SEM ($N = 8$ at control, pH 5.0, 4.0, and 3.0; $N = 5$ at recovery). Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean.

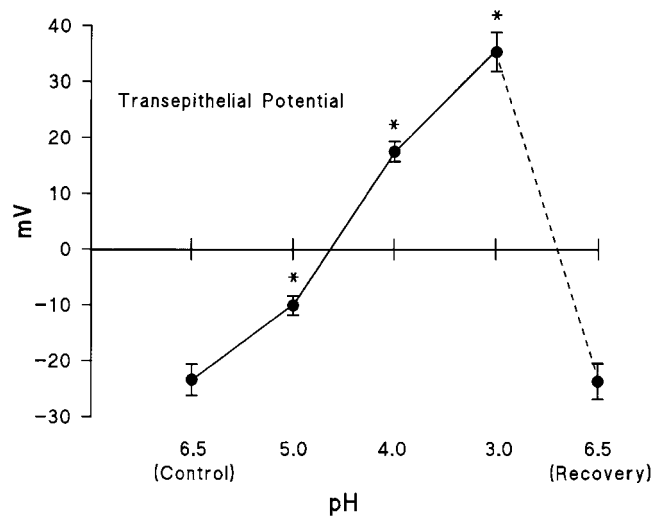


Figure 6. The influence in tambaqui of exposure to a graded low-pH and recovery regime (1-d steps) on the transepithelial potential between the blood and the external water (taken as reference zero). Values are means \pm 1 SEM ($N = 8$ at control, pH 5.0, 4.0, and 3.0; $N = 5$ at recovery). Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean.

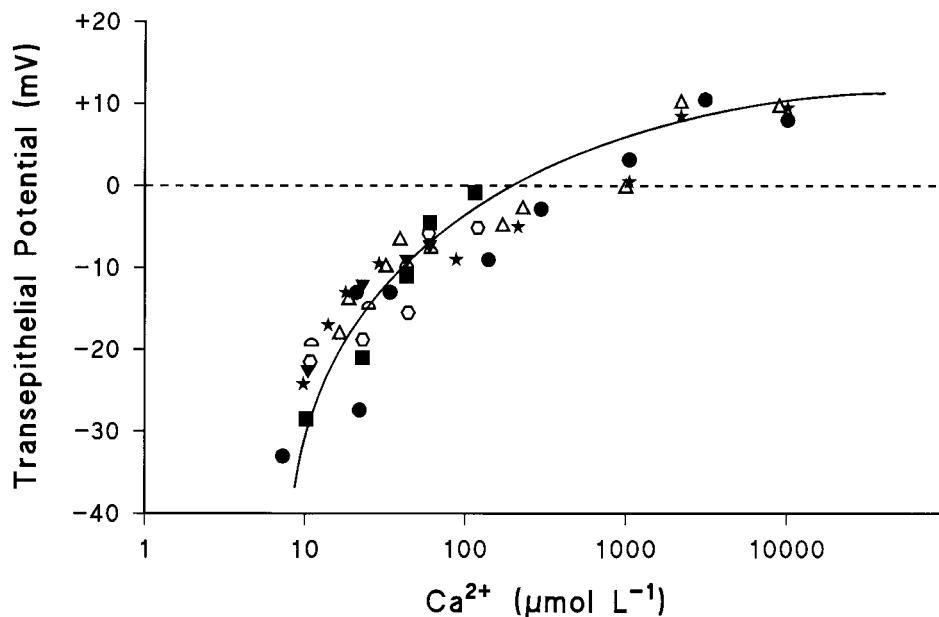


Figure 7. The influence in tambaqui of acute changes in water $[Ca^{2+}]$ (note logarithmic scale) on the transepithelial potential between the blood and the external water (taken as reference zero). Each symbol represents a different fish; $N = 7$.

ing plasma Na^+ and Cl^- and increasing plasma protein levels (Fig. 5).

These effects correlate well with data from a parallel series of flux experiments performed on a separate batch of tambaqui with a similar graded low-pH exposure regime (R. W. Wilson, C. M. Wood, R. G. Gonzalez, M. L. Patrick, H. L. Bergman, A. Narahara, and A. L. Val, unpublished results). These experiments show that initial net Na^+ and Cl^- losses to the water at pH 4.0 were corrected during continued exposure, whereas larger and persistent losses occurred at pH 3.5. Interestingly, while there was no mortality and full recovery of net ion uptake upon return to control pH after 1 d at pH 3.5, there was some mortality and no restoration of plasma Na^+ and Cl^- levels in the present study after 1 d at pH 3.0 (Fig. 5). This suggests that the threshold for lethal damage to gill transport mechanisms and/or permeability in tambaqui lies between 3.0 and 3.5.

An important point of agreement with earlier studies on species such as the rainbow trout (see McDonald et al. 1980; Wood 1989) is the almost complete absence of blood acid-base disturbance, despite a 5-pH-unit difference between water and blood pH (Fig. 2). In the trout, this stability of acid-base status has been related to the fact that when exposures are performed in soft water very low in $[Ca^{2+}]$ ($<200 \mu mol L^{-1}$, as in the present study), there is negligible net uptake of acidic equivalents from the external environment (or even slight net base uptake, equivalent to acid excretion at the gills).

In trout, Wood (1989) related the acid-base responses occurring at low environmental pH to the difference between net

Na^+ and net Cl^- fluxes through the constraints of electrical neutrality at a macro level, as explained by the Strong Ion Difference approach (Stewart 1978, 1983). From this viewpoint, the marked blood acidosis commonly seen in trout at high water $[Ca^{2+}]$ was due to a large measured influx of acidic equivalents, constrained by an excess of net Na^+ over net Cl^- loss. At low water $[Ca^{2+}]$, although both Na^+ and Cl^- loss rates increased, Cl^- loss became equal to or slightly greater than Na^+ loss, thereby preventing net acid uptake or promoting slight net acid excretion. However, in the tambaqui and other Amazonian blackwater species, net flux measurements showed that net Na^+ loss consistently exceeded net Cl^- loss at low pH in very soft water, both in the present investigation (Fig. 9) and in the study by Gonzalez et al. (1998). This suggests that other (unmeasured) cations are entering or anions are leaving the fish. Clearly, it would be informative in future studies to measure net acidic equivalent and ion flux rates, together with blood acid-base status, during exposure of tambaqui to low pH in both high- and low- $[Ca^{2+}]$ water.

A notable finding was the complete absence of respiratory disturbance at low pH, whereas nonacidophilic species generally suffer a suffocation response because of gill structural damage, edema, and mucification at pH's below 4.0 (reviewed by Fromm [1980]; Ultsch et al. [1981]; Wood and McDonald [1982]). Even though some measurements were taken in tambaqui close to death at pH 3.0, there was no evidence of any disturbance of arterial blood PO_2 or PCO_2 levels (Figs. 1, 2), lactate elevation (Fig. 4B), or visible signs of hyperventilation. Presumably, the gill surface of the tambaqui is structurally resistant and/or does not show an inflammatory response at severely low pH.

Stress responses (elevated plasma cortisol, glucose, and ammonia levels; Fig. 4), though occurring at only a very low pH

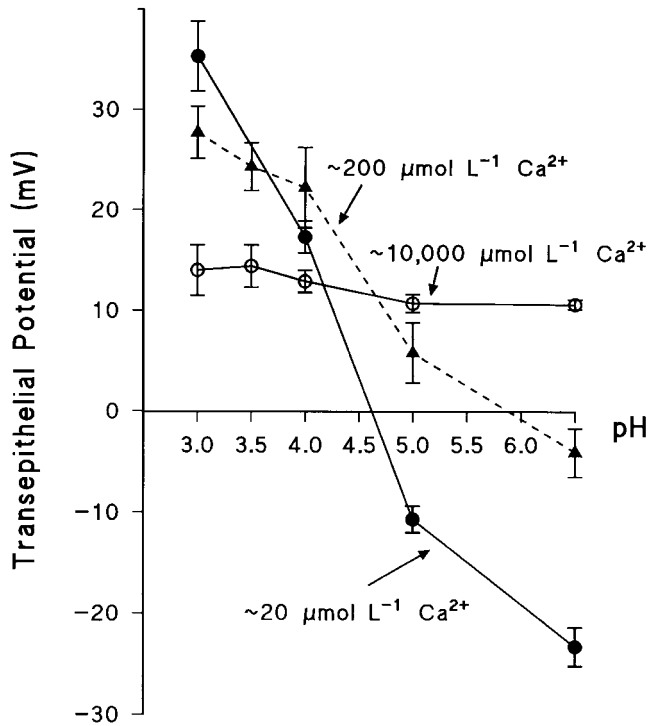


Figure 8. The influence in tambaqui of three different water $[\text{Ca}^{2+}]$'s on the responses of transepithelial potential between the blood and the external water (taken as reference zero) to acute changes in water pH. Values are means \pm 1 SEM ($N = 6$ at $\sim 20 \mu\text{mol L}^{-1}$; $N = 3$ at $\sim 200 \mu\text{mol L}^{-1}$; and $N = 5$ at $\sim 10,000 \mu\text{mol L}^{-1}$ Ca^{2+}).

threshold, were fairly typical of those seen in other teleosts during acid exposure. The well-known effect of cortisol in promoting proteolysis (Anderson et al. 1991; van der Boon et al. 1991) was undoubtedly an important contributor to the persistent elevation in plasma ammonia concentration. The role of cortisol in glucose mobilization remains controversial (see, e.g., Anderson et al. 1991). It is quite possible that catecholamine mobilization may also have occurred, as it does in rainbow trout at pH 4.0 (Ye et al. 1991), and made an important contribution to glucose elevation, either alone or in combination with cortisol (Reid et al. 1992; Perry and Reid 1993).

The general levels of nucleoside phosphates in the erythrocytes of tambaqui were comparable to those recorded previously in this species, with guanylates clearly dominating over adenylates (Val and Almeida-Val 1995; Val et al. 1998). However, to our knowledge, the present data (Fig. 3) are the first to record the effects of low pH exposure on RBC nucleoside phosphate levels in any teleost. They reveal a marked elevation of both guanylates and adenylates, with changes in GTP and ATP being most prominent. These organic phosphates are powerful negative allosteric modifiers of Hb O_2 affinity, so the probable consequence was a rightward shift in the blood O_2 dissociation curve, which would tend to impede O_2 loading at

the gills yet favour O_2 unloading at the tissues. The role of this strategy during low pH exposure is unclear, but it again confirms that these fish had no difficulty with branchial O_2 loading. We cannot eliminate the possibility that these were responses to blood sampling rather than to low pH, because similar responses have been demonstrated after repetitive blood removal, resulting in anemia in other species; the immature erythrocytes that are recruited have higher activities of oxidative phosphorylation (reviewed by Val et al. [1994]). However, it is important to note that blood sampling did not cause anemia in these acid-exposed tambaqui, probably because of simultaneous hemoconcentration (see, e.g., Figs. 1C, 5B) and/or mobilization of erythrocytes stored in the spleen (Milligan and Wood 1982).

Transepithelial Potential Responses of Tambaqui to Low pH

There was a marked and persistent reversal of transepithelial potential in tambaqui from highly negative inside at pH 6.5 to highly positive inside at pH 4.0, in very soft water (Figs. 6, 7). These responses were very similar, both qualitatively and quantitatively, to the detailed observations of McWilliams and Potts (1978) on brown trout in Ca^{2+} -free water. The pattern also parallels less detailed results on rainbow trout at low pH in soft water (Ye et al. 1991). However, the explanation remains unclear.

Most workers agree (though not all; see below) that at circumneutral pH, the inside negative transepithelial potential represents a diffusion potential due to the differential perme-

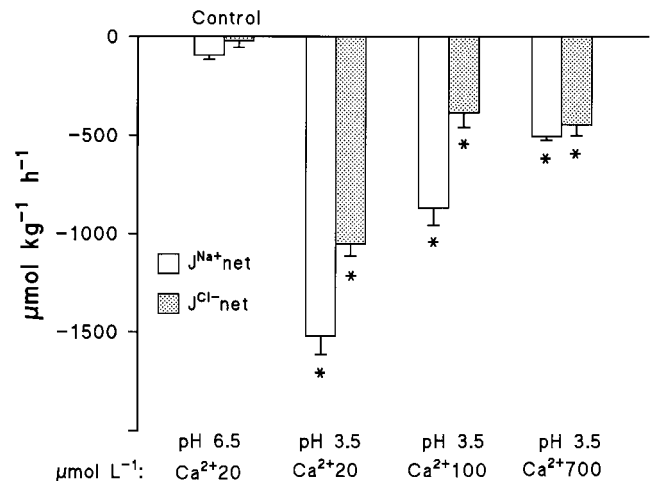


Figure 9. The influence in tambaqui of three different water $[\text{Ca}^{2+}]$'s on the net flux rates of sodium ($J^{\text{Na}^+}_{\text{net}}$) and chloride ($J^{\text{Cl}^-}_{\text{net}}$) to the external water during a 1-h acute exposure to pH 3.5 ($N = 6$ for all treatments). The control flux data shown at pH 6.5 represent data from one experiment ($[\text{Ca}^{2+}] = 20 \mu\text{mol L}^{-1}$ throughout, $N = 6$); control data (all at $[\text{Ca}^{2+}] = 20 \mu\text{mol L}^{-1}$) for the other experiments were virtually identical. Asterisks indicate means significantly different ($P \leq 0.05$) from the respective control mean.

ability of the gills to Na^+ versus Cl^- (Na^+ permeability $>$ Cl^- permeability; see, e.g., Potts [1984]). McWilliams and Potts (1978) and Potts and McWilliams (1989) argued that the transepithelial potential became positive at low pH because of a very high permeability of the gills to H^+ ions and, therefore, a large net entry of H^+ ions from the acidic environment. However, direct H^+ flux measurements (discussed earlier) have demonstrated that this is not the case, at least in salmonids (Wood 1989). McWilliams and Potts (1978) based their calculations on the assumption that Cl^- permeability does not change at low pH, whereas unidirectional flux measurements have shown that Cl^- permeability in fact increases greatly in salmonids (Wood 1989). A more reasonable explanation would be that Cl^- permeability increases to a greater extent than Na^+ permeability at low pH, causing reversal of the potential—that is, a simple pH-dependent modification (Na^+ permeability $<$ Cl^- permeability) of the diffusion potential.

In contrast, Kirschner (1994) described an analogous situation in freshwater crayfish, presented unidirectional flux evidence against the diffusion potential hypothesis, and argued that an electrogenic mechanism responsible for inward Ca^{2+} transport might provide the origin of the transepithelial potential. Alternatively, Randall et al. (1996) have suggested that the negative potential inside is an electrogenic potential, and that its reversal at low pH is due to inhibition of the driving force, an H^+ -extruding pump on the apical surface of the gill cells. To date, available evidence suggests that an H^+ pump may exist at the gill surface and contribute a potential across the apical cell membrane, but it is unclear whether it could create a potential across the whole epithelium (Potts 1994; Lin and Randall 1995).

The Influence of $[\text{Ca}^{2+}]$ on the Responses of Tambaqui to Low pH

The present results provide abundant evidence that the gills of tambaqui are extremely sensitive to water $[\text{Ca}^{2+}]$. Log scale increases in $[\text{Ca}^{2+}]$ drove the transepithelial potential to positive values at circumneutral pH (Figs. 7, 8), a phenomenon that has now been documented in many other freshwater fish and crustaceans (reviewed by Kirschner [1994]). Depending on one's view of the origin of the transepithelial potential (see above), this could be explained as an effect of $[\text{Ca}^{2+}]$ on one or more of three properties: on the Na^+ permeability to Cl^- permeability ratio, on Ca^{2+} transport, or on the H^+ -pump. Elevated $[\text{Ca}^{2+}]$ also attenuated or prevented the marked changes in transepithelial potential that occur at low pH (Fig. 8), results very similar to those reported by McWilliams and Potts (1978) on brown trout. At present, we favour the simplest explanation, that low external pH (high $[\text{H}^+]$) and high external $[\text{Ca}^{2+}]$ both act to shift the Na^+ permeability to Cl^- permeability ratio below unity, thereby reversing and stabilizing the diffusion potential. For example, both agents could act by titrating negative charge on paracellular channels, thereby modi-

fying their permselectivity to Na^+ versus Cl^- in a similar fashion while having very different effects on absolute permeabilities (i.e., H^+ increasing and Ca^{2+} decreasing absolute permeabilities). Simultaneous electrical and radioisotopic (unidirectional) flux measurements, as opposed to the “cold” net flux measurements of the present study (Fig. 9) will be required to test these ideas.

Nevertheless, the present net flux measurements do confirm a marked protective effect of elevated water $[\text{Ca}^{2+}]$ against the net losses of Na^+ and Cl^- that occur at low pH (Fig. 9). Again, the action of $[\text{Ca}^{2+}]$ appears similar to that seen in salmonids (McDonald 1983; Freda and McDonald 1988; Wood 1989) and differs from the insensitivity to $[\text{Ca}^{2+}]$ seen in both the acidophilic perch (Freda and McDonald 1988) and three blackwater species collected from the Rio Negro (Gonzalez et al. 1998). Direct comparison tests showed that all three of the latter Rio Negro species lost far less Na^+ , K^+ , and Cl^- when acutely challenged with low pH than did the tambaqui (Gonzalez et al. 1998). Losses by the tambaqui at pH 3.5 were comparable to those of the perch (Freda and McDonald 1988; McDonald et al. 1991).

The responses of the tambaqui to $[\text{Ca}^{2+}]$ at low pH also differed qualitatively from those of other acidophilic species such as the banded sunfish. The sunfish shows an extremely high affinity of the gill for $[\text{Ca}^{2+}]$, with protective effects against low pH complete by 100–125 $\mu\text{mol L}^{-1}$ (Gonzalez and Dunson 1987, 1989a). In the present study, protective effects were seen at $[\text{Ca}^{2+}]$'s up to 700 $\mu\text{mol L}^{-1}$ (Fig. 9), comparable to the range seen in acid-sensitive species such as trout and shiners (Freda and McDonald 1988) and the moderately acid-tolerant Amazonian blackskirt tetra (Gonzalez et al. 1997). Unfortunately, the picture is further clouded by the observation that lanthanum, thought to be a powerful antagonist that displaces Ca^{2+} from tight junctions (Weiss 1974), had no effect on Na^+ or Cl^- losses in the tambaqui, whereas it augments loss rates in trout, shiner (Freda and McDonald 1988), and blackskirt tetra (Gonzalez et al. 1997). These results underscore both the diversity of strategies for living at low environmental pH in various species and the incomplete nature of our current understanding of gill $[\text{Ca}^{2+}]$, $[\text{H}^+]$, and ion flux relationships. Nevertheless, it is clear that many of the basic principles with respect to responses to low pH first elucidated in salmonids apply in tambaqui, and that most differences are quantitative rather than qualitative.

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