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Calcium fluxes in *Hoplosternum littorale* (tamoatá) exposed to different types of Amazonian waters

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Fishes that live in the Amazonian environment may be exposed to several kinds of waters: “black waters”, containing high dissolved organic carbon and acidic pH, “white waters”, with ten fold higher Ca^{2+} concentrations than black waters and neutral pH, and “clear waters”, with two fold higher Ca^{2+} concentrations than black waters and also neutral pH. Therefore, the aim of the present study was to analyze Ca^{2+} fluxes in the facultative air-breather *Hoplosternum littorale* (tamoatá) exposed to different Amazonian waters. Fishes were acclimated in well water (similar to clear water) and later placed in individual chambers for Ca^{2+} fluxes measurements. After 4 h, water from the chambers was replaced by a different type of water. Transfer of tamoaatás to ion-poor black or acidic black water resulted in net Ca^{2+} loss only in the first 2 h of experiment. However, transfer from black or acidic black water to white water led to only net Ca^{2+} influxes. The results obtained allowed us to conclude that transfer of tamoaatás to ion-poor waters (black and acidic black water) led to transient net Ca^{2+} loss, while the amount of Ca^{2+} in the ion-rich white water seems adequate to prevent Ca^{2+} loss after transfer. Therefore, transfer of tamoaatás between these Amazonian waters does not seem to result in serious Ca^{2+} disturbance.

Os peixes que vivem na Amazônia são expostos a vários tipos de água: águas pretas, contendo grande quantidade de carbono orgânico dissolvido, águas brancas, com concentração de Ca^{2+} dez vezes maior que as águas pretas e pH neutro, e águas claras, com concentração de Ca^{2+} duas vezes maior que as águas pretas e pH também neutro. Dessa forma, o objetivo deste trabalho foi analisar o fluxo de Ca^{2+} no peixe de respiração aérea facultativa *Hoplosternum littorale* (tamoatá) exposto a diferentes tipos de águas amazônicas. Os peixes foram aclimatados em água de poço artesiano (semelhante à água clara) e depois colocados individualmente em câmaras para medir o fluxo de Ca^{2+} . Após 4 h, a água das câmaras foi trocada por um tipo diferente de água. A transferência do tamoaatá das águas pobres em íons água preta e preta ácida ou da água branca, rica em íons, para as águas preta e preta ácida, pobres em íons, resulta em uma perda de Ca^{2+} apenas nas duas primeiras horas de experimento. Entretanto, a transferência da água preta e preta ácida, para a água branca resulta em um influxo de Ca^{2+} . Os resultados obtidos nos permitem concluir que a transferência do tamoaatá para as águas preta e preta ácida, pobres em íons, leva a uma temporária perda de Ca^{2+} , e a quantidade de Ca^{2+} na água branca, rica em íons, é adequada para prevenir sua perda após a transferência. Sendo assim, a transferência do tamoaatá entre as águas estudadas não resulta em sérios distúrbios no Ca^{2+} .

Key words: Ion flux, Negro River, Amazon River, Acidic water.

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Introduction

Tributaries in the Amazon basin exhibit a range of different chemical characteristics. They were classified into three major types on the basis of their appearance as “white waters”, “clear waters” and “black waters” (Gibbs, 1972). Two major rivers the Solimões River and the Negro River join at Manaus to form the Amazon River. The white water (brownish in reality) of the Solimões River has a high amount of suspended sediments (Tardy *et al.*, 2005), neutral pH and is comparatively rich in ions (Aucour *et al.*, 2003). The Negro River is called “black water river”, with much lower ion concentrations (5-20 fold lower than white water), pH of 5.0-6.0 and greater amounts of dissolved organic matter (Aucour *et al.*, 2003; Mortatti & Probst, 2003). In small streams or flooded forests of the Negro River basin the high concentrations of humic and fulvic acids formed in the soil through the decomposition of organic matter can reduce water pH down to 3.0-4.0 (Küchler *et al.*, 2000; Matsuo & Val 2003). Clear water is found in few rivers like the Tapajós River and has two fold higher Ca^{2+} concentrations than black waters and neutral pH (Konhauser *et al.*, 1994).

Normally the floodplain Amazon lakes are supplied by waters from small acidic black water streams year round and by the white waters of the Amazon River during the rainy season. Therefore several Amazon fishes are exposed to seasonal changes from white to black waters and vice-versa year around in the lakes (Araújo-Lima & Goulding, 1997; Barthem & Goulding, 1997) and a few even enter the acidic black waters of the small streams or flooded forests or migrate between those different types of water (Araújo-Lima & Goulding, 1997).

Several studies of ion fluxes between the fishes and their external media were performed in samples collected from the Negro River (Gonzalez *et al.* 1997, 1998, 2002; Wood *et al.*, 1998; Wilson *et al.*, 1999; Gonzalez & Wilson, 2001) and some demonstrated that they are more resistant to acidic waters than those that do not live in black waters (Wilson *et al.*, 1999; Gonzalez *et al.* 1997, 2002). However, only two studies analyzed Ca^{2+} fluxes in Amazon fishes, and they were performed with modified well water, which is similar to the “clear water” (Wilson *et al.*, 1999; Matsuo *et al.*, 2005). In addition, transfer of tamoatá (*Hoplosternum littorale*) and pirarucus (*Arapaima gigas*) from black water to white water or vice-versa induced only minor changes on net Na^+ , K^+ and Cl^- fluxes (Baldissarro *et al.*, 2008), but Ca^{2+} fluxes were not studied. In addition, waterborne Ca^{2+} uptake occurs much slower than Na^+ uptake (Matsuo *et al.*, 2005). Calcium is essential to fish for several biological processes such as bone construction, blood coagulation, and many other cellular functions (Flik *et al.*, 1995). Plasma Ca^{2+} is regulated by food sources or by branchial absorption (Flik & Verbost, 1995). In teleost fish in general 99% of the internal sources of Ca^{2+} are incorporated into bone, scales, teeth and otoliths, and the readily exchangeable Ca^{2+} in the bone of Mozambique tilapia (*Oreochromis mossambicus*) maintained at $200 \mu\text{mol.L}^{-1}$ Ca^{2+} (concentration similar to white water) is around 19% (Flik *et*

al., 1986). The same authors also verified that waterborne Ca^{2+} levels influenced Ca^{2+} fluxes in this species. Therefore, the aim of the present study was to analyze net Ca^{2+} fluxes in tamoatá exposed to different types of Amazonian waters.

Material and Methods

Experimental animals and management conditions. Tamoatá juveniles (9-26 g) were raised in a 200-m^3 earth pond at the Embrapa fish culture sector (Manaus, Amazonas, Brazil). One month before the experiments fish were transferred to a 2000 L indoor fiberglass tank and fed three times a day with a commercial pelleted feed with 45% crude protein (TR 45; Nutron, São Paulo, Brazil). The earth pond at Embrapa and the indoor tank were supplied with well water (26-27°C) ($\mu\text{mol.L}^{-1}$): $[\text{Na}] - 48.6$, $[\text{K}] - 39.6$, $[\text{Ca}] - 32.0$, $[\text{Cl}] - 55.2$, $\text{pH} = 5.5$.

Ca^{2+} flux measurements. After acclimation to these pond conditions, randomly selected fish were placed in individual chambers (420 mL) containing one of three water chemistries; i) black water ($\mu\text{mol.L}^{-1}$): $[\text{Na}] - 24.7$, $[\text{K}] - 12.2$, $[\text{Ca}] - 26.6$, $[\text{Cl}] - 29.0$, $\text{pH} = 5.7$, ii) black water adjusted to pH 3.5 with $0.5 \text{ M H}_2\text{SO}_4$, or iii) white water ($\mu\text{mol.L}^{-1}$): $[\text{Na}] - 135.4$, $[\text{K}] - 25.8$, $[\text{Ca}] - 248.5$, $[\text{Cl}] - 116.8$, $\text{pH} = 7.0$. Black water was collected in the Negro River and white water in the Solimões River, both places near Manaus. Dissolved organic matter is 15.5 mg.L^{-1} in both black water and acidic black water (Gonzalez *et al.*, 2002) and 2.0 mg.L^{-1} in well water and white water (Wood *et al.*, 1998). This first exposure lasted four hours, and then the water in the chambers was replaced by a different type of water and the fish remained in the chamber for an additional 4 h (second exposure). The different water combinations used are listed in Table 1. The control group was submitted to the same handling procedure, but remained in well water for the 8 h experimental period. Water temperature, pH (using a pH100, YSI Inc., Yellow Springs, OH, USA) and dissolved oxygen levels (using a YSI DO200 oxygen meter) were measured. Water pH in the chambers was adjusted only at the beginning of each exposure and measured at the beginning and the end of the second exposure (4 h) to the different waters, and mean maximum differential between initial and final pH readings were 0.17, 0.18, -0.35 and 0.75 units in the black water and acidic black water, white water and well water, respectively.

Table 1. Different types of Amazonian waters where *Hoplosternum littorale* was exposed for determination of ion fluxes. There were two successive measurement periods of 2 h in each exposure. N = 10 for each treatment.

First exposure (4 h)	Second exposure (4 h)
black water pH 5.7	white water pH 7.0
black water pH 5.7	black water pH 3.5
black water pH 3.5	black water pH 5.7
black water pH 3.5	white water pH 7.0
white water pH 7.0	black water pH 5.7
white water pH 7.0	black water pH 3.5
well water pH 5.5	well water pH 5.5

Water temperature in the chambers was 25–27°C and dissolved oxygen levels 4–7 mg L⁻¹. Water samples (10 mL) were taken from the chambers at the beginning and every 2 h up to the end of the experiment and stored in the -20°C freezer for analysis of Ca²⁺ concentrations. The fish were weighed at the end of the experiment.

Water Ca²⁺ levels were measured directly with an AA-1475 atomic absorption spectrophotometer (AAS 11 VARIAN, Australia, Ca²⁺ sensitivity 3.0 µmol.L⁻¹) after addition of a concentrated solution of lanthanum chloride (yielding a solution of 0.2% lanthanum chloride) to reduce interferences in the measurement. Net Ca²⁺ fluxes were calculated according to Gonzalez *et al.* (1998) $J_{net} = V([ion]_1 - [ion]_2) \cdot (Mt)^{-1}$. Where [ion]₁ and [ion]₂ are the bath ion concentrations at the beginning and end of the flux period (every two hours), respectively, V is the bath volume (in liters), M is the mass of the fish (in kg), and t is the duration of the flux period (in hours).

Statistical analysis. Homogeneity of variances was verified by the Levene test. Variances were not homocedastic and therefore net ion fluxes and water pH changes due to different treatments and times were compared using the Kruskal-Wallis ANOVA and Mann-Whitney tests, with the aid of the software Statistica 5.1. Data were expressed as mean ± SEM, and the minimum significance level was P < 0.05.

Results

First exposure. Tamoatás transferred from the tanks of ion-poor well water to flux chambers containing ion-poor acidic black water showed significantly greater Ca²⁺ loss than those transferred to the other waters during the first two hours of exposure. Fish transferred from ion-poor black water and those maintained in well water presented low net Ca²⁺ efflux, while those transferred to white water showed net Ca²⁺ influx. Through the 2–4 h period after transfer from the tanks, net Ca²⁺ efflux of tamoatás maintained in acidic black water reduced to zero and those kept in black water and well water presented low net influx (Figs. 1 and 2), whereas fish in white water increased their net Ca²⁺ influxes by more than three-fold (Fig. 2).

Second exposure. Specimens maintained in well water in the first exposure and with renewal of this well water in the second exposure did not show any significant change in net Ca²⁺ flux up to end of the experiment (Fig. 1). Transfer of tamoatás from black water to acidic black water led to net Ca²⁺ efflux in the first 2 h, but as in the first exposure, at the end of 4 h reversed to a Ca²⁺ influx (Fig. 3a). On the other hand, fish transferred from acidic black water to black water did not show any significant change in net Ca²⁺ fluxes in the first 2 h, but in the 2–4 h period there was a net Ca²⁺ influx (Fig. 3b). Tamoatás transferred to white water irrespective of previous exposure (black water or acidic black water), presented a significant increase of the net Ca²⁺ influxes compared to the fluxes in the previous water type, which then remained constant through the 4 h of the second exposure (Fig. 3a-b). Transfer from

white water to black water or acidic black water led to net Ca²⁺ effluxes in the first 2 h of exposure, and which were reversed to net influx (black water) or reduced to zero (acidic black water) during the 2–4 h period (Fig. 3c).

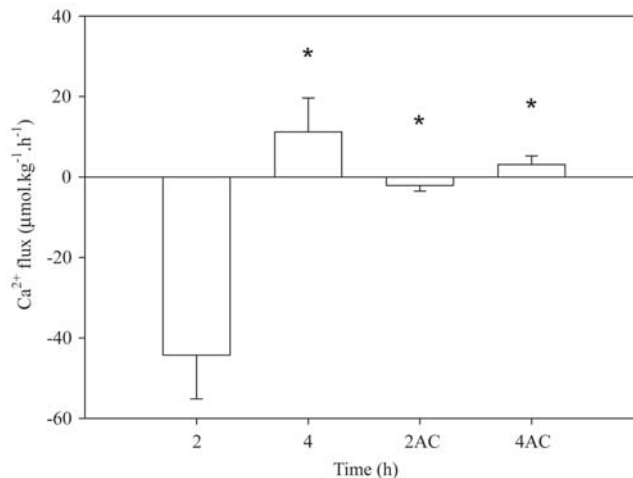


Fig. 1. Net Ca²⁺ fluxes of *Hoplosternum littorale* exposed to well water as a function of time after transfer from tanks of ion-poor well water. Data expressed as mean ± SEM. Positive values indicate net influxes and negative values net effluxes. AC - after change. Asterisks (*) indicate significantly different from 2 h by Kruskal-Wallis ANOVA and Mann-Whitney test (P < 0.05).

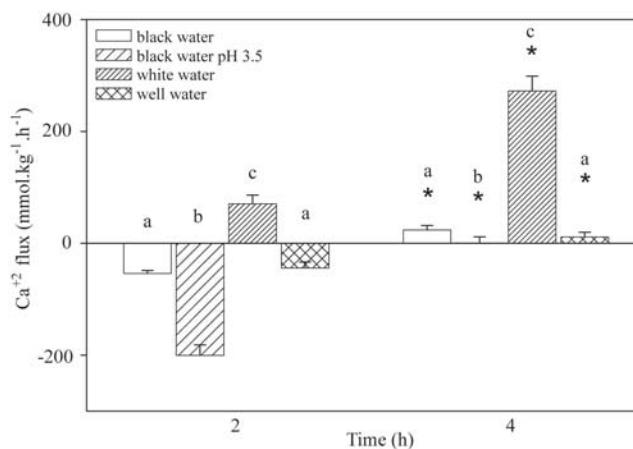


Fig. 2. Net Ca²⁺ fluxes of *Hoplosternum littorale* transferred from tanks of ion-poor well water to experimental flux chambers containing different Amazonian waters. The first four bars show fluxes measured over the first two hours, and the second set of four bars show data for the 2–4 h period after initial transfer. Data expressed as mean ± SEM. Positive values indicate net influxes and negative values net effluxes. Different letters indicate significant differences among groups in the same period of time by Kruskal-Wallis ANOVA and Mann-Whitney test (P < 0.05). Asterisks (*) indicate significantly different from the same group at 2 h (P < 0.05)

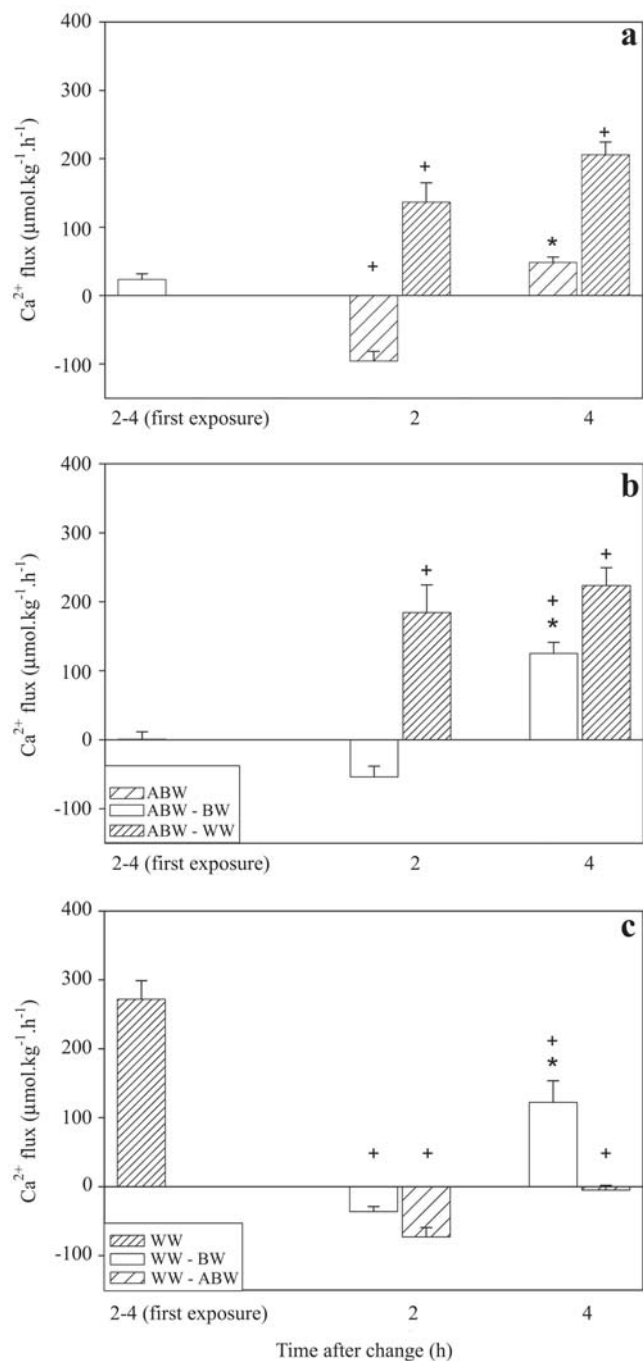


Fig. 3. Net Ca²⁺ fluxes of *Hoplosternum littorale* transferred from black water (BW) to acidic black water pH 3.5 (ABW) or white water (WW) (a), and fish transferred from ABW to BW or WW (b) and fish transferred from WW to BW or ABW (c). The first bar in the left side of the figure represents the 2-4 h flux of the first exposure. Data expressed as mean \pm SEM. Positive values indicate net influxes and negative values net effluxes. Asterisks (*) indicate significantly different from the same group 2 h after transfer by Kruskal-Wallis ANOVA and Mann-Whitney test ($P < 0.05$). Crosses (+) indicate significantly different from the previous water condition by Kruskal-Wallis ANOVA and Mann-Whitney test ($P < 0.05$).

Discussion

The mitochondria-rich cells, located in the gill epithelia, are probably the most important site for Ca²⁺ uptake in fish. The intracellular Ca²⁺ concentration of the chloride cells is very low, and consequently apical uptake is diffusional by a Ca²⁺ channel even from soft water (Evans *et al.*, 2005). The exit of Ca²⁺ across the basolateral membrane is active, involving the Ca²⁺-ATPase and the Na⁺/Ca²⁺ exchanger (Guerreiro & Fuentes, 2007). Tamoatás transferred to chambers with ion-poor black water or well water presented net Ca²⁺ efflux in the first 2 h of experiment. This was expected because tamoatás had not been given time to recover from any handling stress after transfer to the chambers, which is very likely to result in net ion loss (Postlethwaite & McDonald, 1995). Stress increases gill blood flow and paracellular permeability, resulting in ion loss (Cech *et al.*, 1996). Recovery is variable depending on the intensity of stress and species (Gonzalez & McDonald, 1994; Baldisserotto & Val, 2002; Rosso *et al.*, 2006). In the present experiment apparently stress was reduced after 2 h, since net Ca²⁺ fluxes in tamoatás in black water and well water became net influxes, and values were similar to those determined by Wilson *et al.* (1999) for the same species and *Brycon erythropterus* (= *Brycon cephalus*) exposed to well water at pH 6.0. Net Ca²⁺ loss was significantly higher in tamoatá exposed to acidic black water, which was also expected because this water induces net Na⁺, Cl⁻ and K⁺ effluxes in this species (Baldisserotto *et al.*, 2008). Tamoatá transferred to ion-rich white water led to net Ca²⁺ influxes, which increased after 2 h. Apparently the approximately 10-fold higher Ca²⁺ concentration in white water (compared to both black water and well water) was enough to avoid net Ca²⁺ loss or increased Ca²⁺ influx. In disagreement with these results, Mozambique tilapia acclimated to water with 200 μmol.L⁻¹ Ca²⁺ presented higher net Ca²⁺ influx compared to those adapted to 800 μmol.L⁻¹ Ca²⁺ (Flik *et al.*, 1986). Probably this difference is due to the fact that in the study of Flik *et al.* (1986) Ca²⁺ fluxes were determined after 10 weeks acclimation, which may allow for increased chloride cell proliferation, and in the present study fluxes were measured 2-4 h after transference. Small changes of waterborne Ca²⁺ are easier to detect by AAS in ion-poor black and well water, but usually more difficult in white water, which contains comparatively higher Ca²⁺ levels. However, net Ca²⁺ fluxes in tamoatá acclimated to white water were high enough to allow measurements with satisfactory precision. Additional experiments with tamoatá acclimated to white water for a longer period of time and with radioactive isotopes must be performed.

Transfer of tamoatás from ion-poor black water and acidic black water or ion-rich white water to ion-poor black water or acidic black water resulted in net Ca²⁺ loss only in the first 2 h of experiment. The same kind of transfers in tamoatás led to net Na⁺, Cl⁻ and K⁺ loss through 4 h (except Cl⁻ and K⁺ in tamoatás transferred from white water to black water) (Baldisserotto *et al.*, 2008). Net Na⁺, Cl⁻ and K⁺ losses after

transfer from black water (pH 5.5) or slightly acid well water (pH 6.5) to acidic black water (pH 3.5-3.75) were also observed by Gonzalez *et al.* (1998), Wood *et al.* (1998) and Wilson *et al.* (1999) in tambaqui (*Colossoma macropomum*) and by (Gonzalez *et al.*, 2002) in *Geophagus* sp. and *Pimelodus* sp. caught in the Negro River. Exposure to very acidic water loosens tight junctions of gill epithelia, which increases ion efflux by a paracellular route (Wood, 2001). However, unlike the patterns for the monovalent ions, there was no obvious trend for net Ca²⁺ fluxes in tamoatá, tambaqui and matrinxã [*Brycon erythropterus* (= *B. cephalus*)] exposed to gradual well water acidification (Wilson *et al.*, 1999).

It seems that adjustment of Ca²⁺ fluxes to these changes in Amazonian waters is faster than the fluxes of other ions in this species. In addition to this hypothesis, tamoatá transferred to ion-poor black water or acidic black water to ion rich white water presented net only net Ca²⁺ influxes, while according to Baldisserotto *et al.* (2008) there was net Na⁺ loss at least in the first 2 h after these transfers.

The results obtained allowed us to conclude that transfer of tamoatá from ion-poor waters (black water and acidic black water) led to transient net Ca²⁺ loss, while the amount of Ca²⁺ in the ion-rich white water seems adequate to prevent Ca²⁺ loss after transfer. Therefore, transfer of tamoatá between these Amazonian waters does not seem to result in serious Ca²⁺ disturbance.

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