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The handling of salt by the neotropical cultured freshwater catfish *Rhamdia quelen*

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ABSTRACT

The jundiá (*Rhamdia quelen*) is a Siluriform native to South America, intensely cultivated in Southern Brazil, and which has been increasingly considered as a potential substitute for the culture of exotic species. Due to this interest, there is a constant need of methods to reduce parasite infestations common to the species. The use of sodium chloride (NaCl) is one of such practices. However, not much is known about the effects of NaCl on the osmotic homeostasis of this and other commercially relevant tropical freshwater species. This study thus aimed at evaluating the effect of adding NaCl (5, 15, or 25 g/L) to the water for 1 h, on the osmoregulation, and capacity for muscle tissue water regulation of the jundiá. In addition, muscle slices were subjected *in vitro* to isosmotic (control, animal in fresh water) and hyper-osmotic (120, 150, and 180% of control) salines, for evaluation of their capacity to control their water content. Some changes have been observed, when fishes submitted to 25 g/L were compared to freshwater controls: plasma ions and osmolality increased, the last from 260±5 to 419±12 mOsm/kg H_2O; plasma glucose increased from 47.8 to 92.8 mg/dL, and muscle water content decreased from 80 to 76.7%; plasma cortisol levels remained unchanged (~150 ng/mL). Branchial carbonic anhydrase activity also increased in 25 g/L, probably as a response to the metabolic acidosis that develops upon severe saline challenges. *In vitro* experiments indicated a high capacity for water content regulation, even when muscle slices were exposed to an 80% increase in osmolality. Exposure of jundiás for 1 h to 25 g/L was indeed harmful to their osmotic homeostasis, also affecting sensory and motor functions (observed as loss of barbel function, and swim impairment). However, the high degree of euryhalinity shown both *in vivo* and *in vitro* by this freshwater siluriform is coherent with its occasional presence in dilute estuarine waters and to the positioning of its family within a group of seawater-related Siluriforms. Thus, a reasonable amount of salt (15 g/L) for an intermediate time period (1 h) could be employed against parasites of the jundiá, while still being harmless to both the jundiás and humans that consume its flesh.

1. Introduction

The jundiá (*Rhamdia quelen*, Quoy and Gaimard, 1824) is a neotropical catfish (Siluriformes) native to South America, which occurs from Southern Mexico down to Argentina. It is a native species with high potential for aquaculture, and has been cultivated in Southern states of Brazil (Gomes et al., 2000; Carneiro et al., 2002, 2005; Tavares-Dias et al., 2002; Golombieski et al., 2003; Barcellos et al., 2004; Schutz and Leuchtenberger, 2006). The production of this species in Brazil has reached a total of 547 tons in 2004, which still represents only 0.2% of the total national production from aquaculture (Boscardin, 2008). It has been increasingly recognized as a feasible alternative for the gradual substitution of the culture of exotic species such as the Nile tilapia and the channel catfish or the common carp (Schulz and Leuchtenberger, 2006). This species shows 1) good development in subtropical and temperate climates, 2) high fertilization and growth rates, 3) an omnivorous feeding habit, and 4) good commercial acceptance both for human consumption and for sports fishing activities (Marchioro and Baldisserotto, 1999; Gomes et al., 2000; Carneiro et al., 2002, 2005; Tavares-Dias et al., 2002; Golombieski et al., 2003; Barcellos et al., 2004; Camargo et al., 2006; Schulz and Leuchtenberger, 2006; Garcia et al., 2007).

Due to the growing interest for this species, there is a constant search of techniques to minimize stress situations that potentially arise during the culture process. The use of salt (sodium chloride) in aquaculture facilities, for the jundiá (Marchioro and Baldisserotto, 1999; Brandão, 2004; Carneiro et al., 2005; Camargo et al., 2006; Garcia et al., 2007) and other freshwater species as well (Selosse and Rowland, 1990; Francis-Floyd, 1995; Tsuzuki et al., 2001; Luz and Portella, 2002; Gomes et al., 2003; Klein et al., 2004; Gomes et al., 2006; Carneiro et al., 2007), has been one of such practices. The added salt reduces the osmotic gradient between the fish internal fluids and external water, stimulates mucous production, reduces fish agitation in the tank, apparently exerting an anesthetic effect, thus reducing stress mainly during the transportation of young and adult fish (Francis-Floyd, 1995; Marchioro and Baldisserotto, 1999; Tsuzuki et al., 2001; Luz and Portella, 2002; Gomes et al., 2003; Camargo et al., 2006; Gomes et al.,

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2006; Garcia et al., 2007). In addition, it has antiseptic effects, and is specifically effective in the treatment of "Ichthyophthiriasis", that is, in eliminating the ichthyoparasite *Ichthyophthirius multifiliis* (Selosse and Rowland, 1990; Francis-Floyd, 1995; Marchioro and Baldisserotto, 1999; Brandão, 2004; Klein et al., 2004; Carneiro et al., 2005; Camargo et al., 2006; Garcia et al., 2007).

I. multifiliis is a large ciliate protozoan, and one of the most common and relevant pathogens that afflict freshwater fishes. Gill infestation at the trophont stage of the parasite compromises osmoregulation and gas exchange. The most common reported treatments against this pathogen of freshwater fish employ potassium permanganate, copper sulphate, formalin, malachite green, increases in temperature, and sodium chloride (Selosse and Rowland, 1990; Ewing et al., 1994; Buchmann and Bresciani, 1997; Brandão, 2004; Klein et al., 2004; Carneiro et al., 2005; Garcia et al., 2007). Of these treatments, temperature and salt are obviously the only absolutely harmless to human health.

Previous studies on the tolerance of the jundiá, both young and adults, to different concentrations of sodium chloride suggest that, for "quick baths" (15 s), a salt concentration as high as 30 g/L may be employed (Brandão, 2004). However, for longer baths (30 to 60 min), it is necessary that the salt be reduced down to 10–25 g/L (Brandão, 2004; Carneiro et al., 2005); for a chronic exposure of 2 weeks, the concentration of salt proposed is of only 4–5 g/L (Garcia et al., 2007), and even less (1–4 g/L) when an application of unlimited time is intended (Marchioro and Baldisserotto, 1999; Brandão, 2004). Still, it has been reported that even a small amount of salt (1–3 g/L) can lead to mortality of young specimens of jundiá (Golombieski, 2004). Thus, this study aimed at verifying the physiological response of the adult jundiá to different concentrations of sodium chloride (5, 15, and 25 g/L) for an intermediate (thus technically most convenient) time period (1 h), due to still controversial data available for that species, and mainly for the paucity of physiological information for this species in particular and other cultivated freshwater species in general.

2. Materials and methods

2.1. Animals and laboratory maintenance

The 86 specimens of *Rhamdia quelen* (37.7±2.3 g and 15.8±0.5 cm) were all obtained from culture facilities, either from the Catholic University of the state of Paraná (PUC-PR, 25°35′S, 49°13′W), or from "Fazenda Girassol" (26°09′S, 48 52′W), in the state of Santa Catarina, between June 2007 and April 2008. The fish have been transported (for 45–90 min) in local fresh water under aeration or with 100% oxygen pumped to the plastic bag, until reaching the laboratory, in the Department of Physiology of the Federal University of Paraná, in Curitiba. They were acclimated to laboratory conditions in 250 L tanks containing dechlorinated tap water, under constant aeration, biological filtration, and a photoperiod of 12 h light: 12 h dark, for 4–7 days. No mortality occurred during the acclimation period. Fish were fed daily, always in the morning, with a total amount of ~20 g of commercial food available for this species (extruded, Supra, 32% protein). Leftovers (after ~20–30 min) were immediately removed from the tank. The jundiás were maintained in the stock tank at a maximum density of 26 fishes (~16 cm long) in 250 L, thus well below the density considered a limit for similarly-sized individuals of this species (Barcellos et al., 2004), of ~75 fishes/250 L. Temperature of the tank was maintained at 20±2 °C throughout the acclimation period. All control and experimental fishes were not fed for the last 24 h before being sampled.

2.2. Whole-animal experiments and sampling for blood and tissues

Aquaria (20 L) containing water with salt (NaCl, plain ground coarse NaCl commonly used for aquaculture of the jundiá) added at 5, 15, or 25 g/L were prepared approximately 50 hours before receiving a fish. Each aquarium initially received tap water, and remained under aeration for ~36 h for dechlorination. At this time NaCl was added, fully dissolved by agitation, and water salinity was measured (salinometer refractometer Shibuya S28), being respectively of 5, 15, and 25‰. After overnight aeration, salinity was again verified (never was any correction needed). Osmolality, ions, and pH of these waters are provided in Table 1. Only then 1 single fish was placed in the aquarium. This whole procedure was carried out 17 times, independently, for salinities 5 and 15 g/L, and 18 times for salinity 25 g/L. Each fish was then observed for 1 h, and the following aspects were noted: skin color, barbel tactile function, and swim pattern. After this hour of exposure and observation, the fish was anesthesized using benzocaine (80 mg/L). After complete anesthesia, a blood sample was obtained through caudal vein puncture using heparinized (Liquemine®-sodium heparin) insulin syringes, and was immediately centrifuged for 5 min (~3000 xg, at room temperature). Plasma thus obtained was frozen at −20 °C until assayed for ions, osmolality, cortisol, and glucose. Fishes were then very quickly killed by decerebration using a scalpel, and were dissected for tissue sampling (gills, kidney, muscle). All branchial arches were removed. The largest arch (either side) was removed and gill filaments were separated for tissue hydration determination, and the remaining arches were saved for determination of carbonic anhydrase specific activity. A muscle slice of (axial muscle, right side) was dissected for tissue hydration determination, and the whole kidney was removed for the carbonic anhydrase assay. Tissues were immediately frozen at −80 °C. Control individuals (n=18) were sampled directly from the acclimation tank (fresh water); control fish were handled for anesthesia, blood and tissue sampling exactly as the experimental fish. This experimental protocol has been approved by the Institution's Committee on Ethics in Animal Experimentation (certificate number 241, issued on August 9, 2007).

Table 1
Ions, osmolality, and pH measured in a single sample of the control and experimental waters prepared

Water	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	$[K^+]$ (mM)	$[Mg^{2+}]$ (mM)	Osmolality (mOsm/kgH_2O)	pH
0 g/L (control)	0	0.13	0	0.009	23	7.63
NaCl 5 g/L	94	99	0	0.07	190	7.12
NaCl 15 g/L	250	224	0	0.12	466	6.94
NaCl 25 g/L	381	311	0	0.24	771	6.74

2.3. Plasma determinations

Chloride and magnesium ions were assayed using colorimetric methods (commercial kits Labtest®, Brazil), in undiluted samples (spectrophotometer ULTROSPEC 2100 pro-Amersham Pharmacia Biotech, Sweden). Chloride ions react with mercury thiocyanate in the presence of ferric nitrate forming ferric thiocyanate, with absorbance read at 470 nm. Magnesium ions react with sulphonated magon in alkaline medium, forming a pinkish complex, proportional to the total magnesium content of the sample, with absorbance read at 505 nm. Ions sodium and potassium were assayed in diluted samples (1:100, Ultrapure water) through flame photometry (CELM FC-180, Brazil). Plasma osmolality was determined in undiluted samples (vapor pressure microosmometer Wescor® VAPRO 5520, USA). Plasma cortisol was assayed according to the method of Munro and Stabenfeldt (1984). The polyclonal antibody R-4866 produced by the University of California at Davis was diluted (1:8500) in cover solution (pH 9.6). Samples were appropriately diluted (1:32 or 1:64) in phosphate buffer (pH 7.0), to remain within the standard curve range (r^2=0.9754). The conjugate (cortisol-horseradish peroxidase) was diluted 1:20,000. Samples were always assayed in triplicates in microplates of 96 wells, and were read at 405 nm (Elisa Sunrise Tecan Deutschland Gmbh, Germany). Plasma glucose levels were determined through a colorimetric method, also

using a commercially available kit (Labtest®) in undiluted samples, with absorbance read at 505 nm. The enzymatic method employs a coupled reaction of glucose oxidase and peroxidase.

2.4. Tissue determinations

2.4.1. Muscular and branchial hydration

For the quantification of tissue hydration, thawed tissues were weighed (gills 0.1085±0.0126 g, muscle 0.2302±0.0242 g, n=70 for both tissues) — wet weight, inside pre-weighed closed tubes (Balance Bioprecisa FA2104 N, precision 0.1 mg, Brazil), and then dried for 24 h at 60 °C. Dried tissues were then weighed again (dry weight), and the loss in weight (water) was expressed as a percentage of the initial wet weight of the sample.

2.4.2. Carbonic anhydrase specific activity

For the assay of carbonic anhydrase (CA) activity, tissue samples (gills and kidneys) were thawed, weighed (gills 0.1321±0.0022 g, kidneys 0.1737±0.0037 g, n=78 for both tissues) and homogenized at 10% (weight/volume, in g/mL) with phosphate buffer 10 mM, pH 7.4. The homogenate was then centrifuged (~2000 xg for 5 min, room temperature), and the supernatant was aliquoted for the protein and enzyme activity assays. Carbonic anhydrase activity was assayed using the method described by Vitale et al. (1999), based on Henry (1991). Activity was quantified through a reaction medium containing mannitol (225 mM), sucrose (75 mM), and tris-phosphate (10 mM), at pH 7.4. To 7.5 ml of this solution, 50 µl of tissue homogenate was added, and 1 ml of cold (2.5 °C) distilled water saturated with CO_2. Immediately after the addition of CO_2-saturated water, the reduction in pH (inoLAB pH meter Level 1, WTW®) was followed for 20 s, with pH readings every 4 s. The slope of the linear regression (pH against time, with data from 4 to 20 s), for each sample, represented the rate of the catalyzed reaction (CR) by the carbonic anhydrase present in the sample. The R^2 of the regression lines used to derive the rate of catalysis was always above 0.98. The uncatalyzed rate of pH drop was considered as the slope of the regression line obtained in the absence of tissue homogenate, and was always determined at least in quadruplicates, at each session of sample processing. Carbonic anhydrase specific activity (CAA) was calculated as CAA=[(CR/NCR)−1]/mg total protein in the sample (Burnett et al., 1981; Vitale et al., 1999).

Total protein content in tissue homogenates was determined according to Bradford (1976). This method is based on the color change of the reagent Coomassie Brilliant Blue G-250, from red to blue, when bound to proteins. Assays were performed in triplicates for each sample in microplates, employing the reagent Bio-Rad Protein Assay (Bio-Rad Laboratories®), with absorbance read at 620 nm (Elisa Sunrise Tecan Deutschland Gmbh, Germany).

2.5. Isolated muscle: in vitro evaluation of water content regulatory capacity

Sixteen fish were taken directly from the acclimation tank, were anesthesized and sacrificed, as described above. Three similar (0.2141±0.0099 g, n=40) muscle slices were removed from each fish (axial muscle, right side), and were transferred to a 10-mL beaker containing control saline, of osmolality similar to plasma osmolality of the jundiá in fresh water, (Table 2). One of the 3 slices remained in the control saline throughout the experiment, and the other 2 were later transferred to beakers containing experimental salines, hyper-osmotic to the plasma of freshwater jundiás (Table 2). The hyper-osmotic salines represented extracellular osmolality increases of 20 (n=8), 50 (n=8), or 80% (n=8), with respect to control values (Table 2). At the start of the experiment, the muscle slice was blot dried on filter paper, in a systematic way, on both sides (for ~3–4 s), and was then transferred to a beaker (time zero) containing one of the salines, either control or hyper-osmotic (~10 mL, at room temperature), according to Freire et al. (2008). Every 15 min, each muscle slice was again blot dried and weighed, until completing 120 min of exposure to the control or experimental salines.

Table 2
Composition of the control and experimental hyper-osmotic salines used in the *in vitro* experiments with the isolated muscle slices of *Rhamdia quelen*

Salts (mM)	Control or experimental saline			
	Control	120%	150%	180%
NaCl	130	169	208	247
KCl	3	3	3	3
$MgCl_2$	1	1	1	1
$CaCl_2$	2	2	2	2
Measured Osmolality (mOsm/kg H_2O)	274	337	409	487

Additional components, of constant concentration in all salines: glucose (5 mM), $NaHCO_3$ (2 mM), HEPES acid (5 mM) and glycine (5 mM), pH 7.6 (according to Freire et al., 2008).

2.6. Statistics

All data are presented as means±standard error of the means. Results of plasma (ions, osmolality, cortisol, and glucose concentrations) and tissue determinations (muscle and branchial hydration, and branchial and renal CA activity) were analysed using one-way ANOVAs: the factor "salinity" had 4 levels – freshwater control, and salinities 5, 15, and 25 g/L – and was followed by the *post hoc* test of Tukey to localize differences. Branchial and renal CA activity groups, for a same salinity, were in addition compared through unpaired Student's t-tests. Results from the *in vitro* experiment (muscle tissue wet weight), as percentages of initial weight were analysed through one-way repeated measures ANOVAs. The factor "time" had 9 levels: 0, 15, 30, 45, 60, 75, 90, 105, and 120 min, for each control or experimental condition. For each of the 4 time points (excluding time zero), controls and each experimental group were compared through paired Student's t-tests. Whenever data did not meet normality or variances were not homogeneous, the non-parametric test of Kruskal-Wallis was employed. For all analyses, the level of significance considered was of 0.05.

3. Results

3.1. Behavioral observations

In the stock tank, all individuals displayed pale, pinkish skin, due to the whitish, clear colour of the tank. Those fishes transferred to the aquaria with 5 g/L of salt changed their skin colour to a grayish shade, normal for the species. They did not show any behavioral alteration with respect to control fish in fresh water, and maintained a normal swimming pattern, and normal barbel use. The skin of 50% of the individuals exposed to 15 g/L NaCl remained pinkish in the experimental aquaria. The fish remained still on the bottom for most of the time, showing occasional bursts of swimming activity. Barbel function remained normal. All jundiás exposed to 25 g/L of NaCl maintained a pinkish skin, and after approximately 15 min started to display loss of barbel function (hitting the wall of the aquaria) and a very clear change in their swimming pattern. The fish remained on the bottom, still, and the bursts of activity resulted in an erratic swimming pattern with the body vertically-oriented.

3.2. Plasma determinations

Plasma sodium of *R. quelen* remained at approximately 140 mM, in the control fish maintained in fresh water, and in the fish exposed 5 or 15 g/L, and increased only in the group exposed to 25 g/L (181 mM) (Fig. 1A). The same pattern observed for sodium was observed for plasma chloride, with approximately 115 mM of chloride in controls and in the fish exposed to 5 g/L. Plasma chloride increased in fish

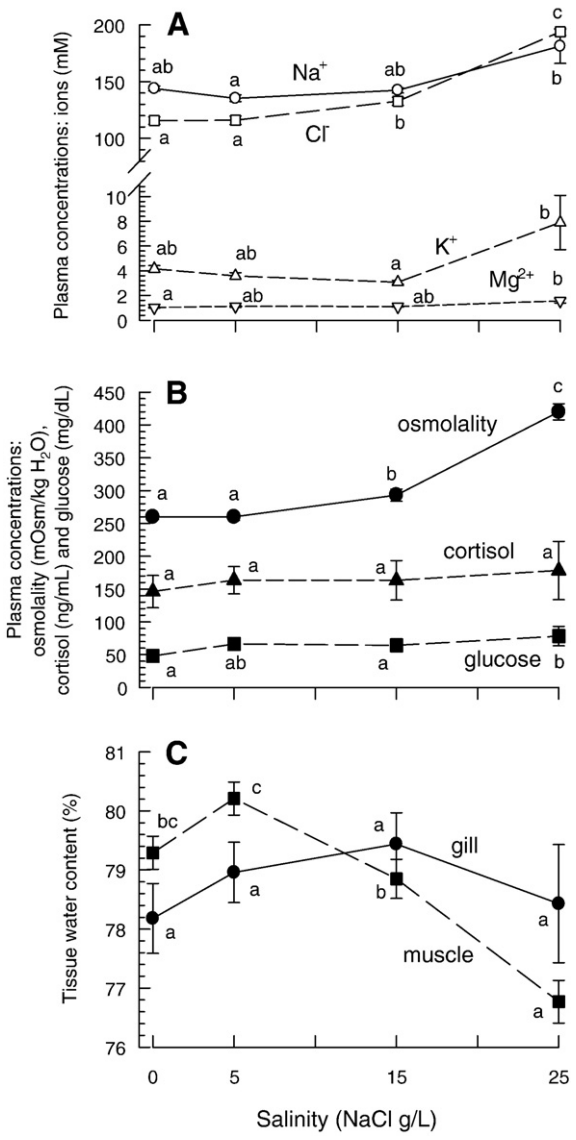

Fig. 1. Plasma concentrations and tissue water content in *Rhamdia quelen*, under control conditions in fresh water (0 g NaCl/L, n=10–26 for the different analyses) or when submitted for 1 h to 5 (n=10–17), 15 (n=10–16), or 25 g/L of NaCl (n=5–13). A: Plasma concentrations of the ions Na^+, Cl^-, K^+, and Mg^{2+} (mM); B: Plasma osmolality (mOsm/kgH_2O), cortisol (ng/mL) and glucose (mg/dL) concentrations; C: muscle and gill water content (%). Within a same line/parameter, symbols that share a same letter represent groups that are not significantly different. When error bars are not visible, they are smaller than the symbol size.

exposed to 15 g/L (133 mM), and a further increase was observed in the jundiás exposed to 25 g/L (194 mM). At this highest salt treatment a cross-over of chloride concentration to higher-than-sodium concentration is noted, with a reduction in the strong ion difference (Fig. 1A). Plasma potassium concentration in *R. quelen* remained stable in controls, and in those maintained in 5 and 15 g/L: 4.1–3.1 mM; in 25 g/L, plasma potassium concentration increased to 7.9 mM (Fig. 1A). Plasma magnesium of *R. quelen* was extremely stable, remaining between 1.02 and 1.12 mM in controls, and in fishes exposed to 5 and to 15 g/L. Again, in 25 g/L, plasma magnesium of the jundiás (1.56 mM) was higher than the values measured in freshwater controls (Fig. 1A).

Plasma osmolality of *R. quelen* was 260 mOsm/kgH_2O in freshwater controls and in those exposed to 5 g/L. It increased in the fish exposed to 15 g/L (293 mOsm/kgH_2O), and had a marked increase in the fish exposed to 25 g/L: 420 mOsm/kgH_2O. This rise in plasma osmolality observed in the jundiás exposed to 25 g/L represented an increase of 62% with respect to controls in fresh water and in those exposed to 5 g/L (Fig. 1B). Plasma cortisol of *R. quelen* ranged between 146 ng/mL in controls and 178 ng/mL in those fishes exposed to 25 g/L. However, there were no differences between controls and all experimental groups (Fig. 1B). Plasma glucose of *R. quelen* also remained stable along the experimental groups, 47.8–64.1 mg/dL in freshwater controls and in those exposed to 5 and 15 g/L. In fishes exposed to 25 g/L (78.2 mg/dL), glycemia was higher than in freshwater controls and in those exposed to 15 g/L (Fig. 1B).

3.3. Tissue determinations

3.3.1. Muscular and branchial hydration

The percentage of water in the muscle of *Rhamdia quelen,* of ~80%, remained rather stable in all groups, until the group of fishes exposed to 15 g/L. A reduction with respect to freshwater controls was noted only in those exposed to 25 g/L: 76.8% (Fig. 1C). In contrast, in the gills the degree of hydration remained at approximately 78–79%, for all groups (Fig. 1C).

3.4. Isolated muscle: evaluation of water content regulatory capacity

The weight of the muscle slices in control saline (osmolality of 274 mOsm/kg H_2O, Table 2) was not stable along the 2 hours of observation, reaching ~104% of their initial weight after 75–120 min

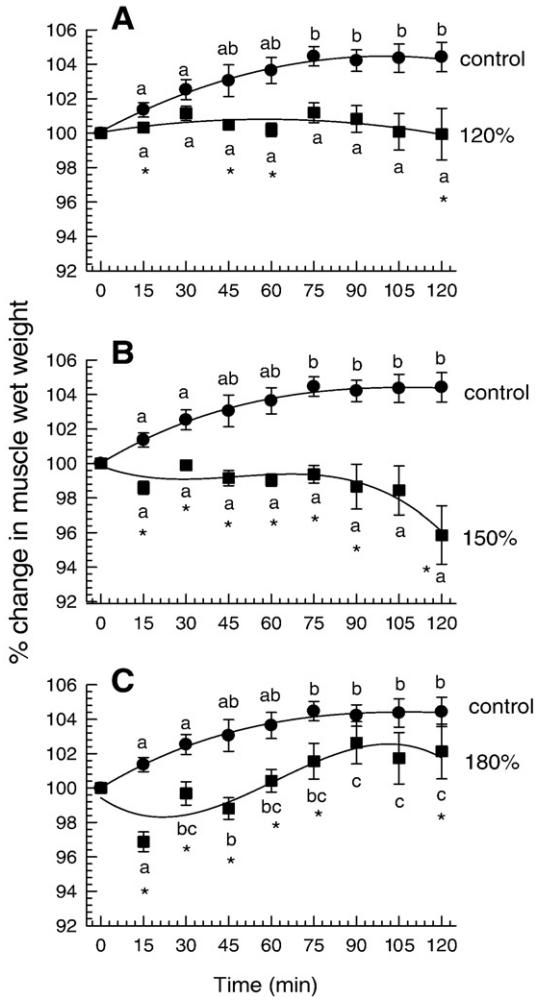

Fig. 2. Time course of the change (%) in the wet weight of muscle slices of *Rhamdia quelen* submitted *in vitro*, for 120 min, to either control (n=16) or hyper-osmotic media (n=8). A: Muscle wet weight in control and 120% saline; B: in control and 150% saline; C: in control and 180% saline. The same controls are repeated in A, B, and C. Within a same line, symbols that share a same letter represent groups that are not significantly different. When error bars are not visible, they are smaller than the symbol size.

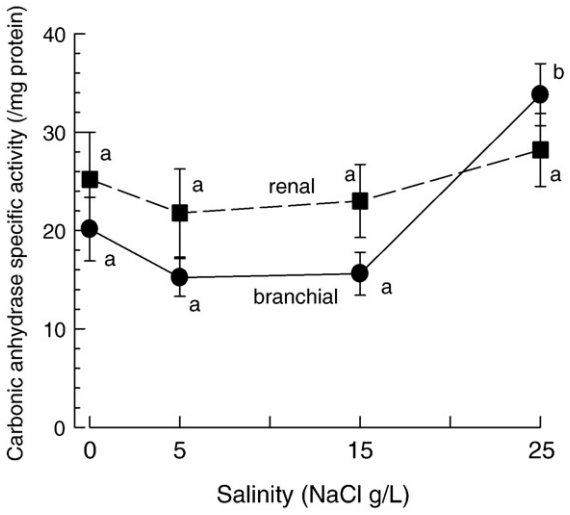

Fig. 3. Branchial and renal carbonic anhydrase specific activity (/mg protein) of *Rhamdia quelen* under control conditions in fresh water (0 g NaCl/L) or when submitted for 1 h to the salinities of 5, 15, or 25 g/L of NaCl. Within the same tissue, symbols that share a same letter represent groups that are not significantly different.

(Fig. 2). The muscle slices submitted to hyper-osmotic salines have shown the capacity to return to their initial levels of hydration after initially losing water (i.e., weight). In the 120% hyper-osmotic saline (337 mOsm/kg H_2O), a slight oscillation was noted (99.9 to 101.1%), but with stability until the end of the experiment, with some values below the controls weighed at the same time (Fig. 2A). In the 150% saline (409 mOsm/kg H_2O), there was no decrease in the hydration level along time, but the values (95.9 to 99.9%) were always below those measured in the controls, except for 105 min (Fig. 2B). In the most concentrated saline (180%, 487 mOsm/kg H_2O), muscle hydration measured at 15 min was the lowest (96.9%), but was followed by an increase along the experiment: 98.8 to 102.6%, reaching values similar to the respective controls in the final phase of the experiment (90 and 105 min) (Fig. 2C).

3.5. Carbonic anhydrase specific activity

The branchial specific activity of the enzyme carbonic anhydrase of the jundiás increased in the fish exposed to 25 g/L of NaCl: 33.8/mg protein, when compared to the previous groups (15.2 to 20.1 /mg protein) (Fig. 3). The renal activity remained unaltered upon all treatments: 21.8 to 28.2 /mg protein (Fig. 3).

4. Discussion

The addition of salt (NaCl) to the water in freshwater fish aquaculture facilities is considered a stress reducer and an antiseptic procedure. However, for freshwater fishes, salt in the water means a challenge to their osmotic homeostasis. Freshwater fish have their body fluids hyper-osmotic (osmolality of 260–330 mOsm/kgH_2O) with respect to their external medium. In this situation there is passive gain of water and loss of ions and, accordingly, use of metabolism to drive ion uptake through their gills and to reabsorb ions from the glomerular filtrate, eliminating a dilute urine. As freshwater fish are transferred to saline water, they will necessarily suffer a disturbance of their usual homeostatic steady-state sustained in fresh water (reviews in Evans, 1993; Bone et al., 1995; Jobling, 1995; Evans et al., 2005). In the experimental protocol used here, the exposure of the jundiás to 25 g/L of common salt (NaCl) used in aquaculture practices dramatically disturbed their osmo-ionic homeostasis, and their responses demonstrated osmotic stress and non-adaptation to the challenge imposed (e.g. Martínez-Álvarez et al., 2002; Sampaio and Bianchini, 2002). Besides increased plasma concentrations, morbidity and evidences of nervous system failure (lack of skin color change upon background change, loss of barbel function, totally disturbed swimming pattern) have also been observed. In addition, the degree of hydration of muscle tissue has decreased. However, despite all these changes, there has been no reduction in the degree of hydration of the branchial tissue. Still, the result observed in the muscle tissue of the jundiás upon the *in vivo* salinity challenge reveal a fair capacity to regulate its water content, even when facing ~62% of increase in plasma osmolality, when fishes were exposed for 1 h to 25 g/L of salt.

These *in vivo* results have been confirmed by the results observed upon the exposure of the isolated muscle slice to the hyper-osmotic salines (*in vitro*), representing a direct hyper-osmotic challenge to the tissue. For all the 3 levels of hyper-osmotic challenge offered to the tissue, after a certain initial (15 min) weight loss, there was always a trend to recover the original weight (i.e., level of hydration), until the end of the experiment, especially in the muscle slices submitted to 180% saline (Fig. 2C). In the first 60 min of the experiment, the response of the muscle of the jundiá was the same as that shown by the isolated muscle of another Siluriform, *Corydoras ehrhardti*, submitted to a 50% hyper-osmotic challenge (150% saline), according to the same experimental procedures employed here (Freire et al., 2008). However, in the second hour of exposure (from 60 to 120 min), the pattern of stability shown here by the muscle of the jundiá was very different from the progressive loss of muscle weight (=loss of water, reaching almost 10% of weight loss with respect to initial weight) observed with the muscle of *C. ehrhardti* (Freire et al., 2008). It should be noted that the weight (hydration) increase observed in the muscle of *Rhamdia quelen* submitted to the control saline was quite unexpected and different from the behavior of muscle slices in control salines, for all the other 9 species previously tested under this protocol (Freire et al., 2008). The first hypothesis to explain this weight increase was that it was a consequence of amino acid uptake (glycine in the saline). But the control trace was exactly the same when the muscle slices of *R. quelen* were submitted to a control saline without glycine (n=8) (osmolality of 263 mOsm/kg H_2O, results not shown). Thus, it seems that the muscle tissue of the jundiá spontaneously takes up solutes and water. In any case, controls were totally reproducible and consistent, and the response to the hyper-osmotic challenges was very clear. It is evident that muscle tissue of *R. quelen* is very effective in solute uptake, and regulatory volume increase.

The capacity to regulate its level of hydration shown by the muscle tissue of *Rhamdia quelen*, even when facing such a huge hyper-osmotic challenge (~60% *in vivo*, 80% *in vitro*), is somewhat surprising, for a fully freshwater teleost. The jundiá belongs to the order Siluriformes, of primary origin and long evolution in fresh water (Ostariophysi) (Nelson, 2006; Froese and Pauly, 2008; Vergara et al., 2008). However, this order displayed vast radiation, probably in the end of the Cretaceous (Vergara et al., 2008), and is in fact essentially freshwater. However, it has 2 families of marine catfishes: Plotosidae and Ariidae. The family Heptapteridae of *R. quelen* is grouped together with those families of marine siluriforms upon phylogenetic analysis (Reis, 1998; Shimabukuro-Dias et al., 2004; Nelson 2006; Froese and Pauly, 2008; Vergara et al., 2008). Indeed, another species of the genus *Rhamdia* (*R. guatemalensis*: Jiménez and Cooke, 2001), and *R. quelen* itself, occur in estuarine habitats (Loebmann and Vieira, 2006; Milani and Fontoura, 2007; Vargas e Bessonart, 2007; Vitule, 2007). The salinity of these estuarine habitats where specimens of *R. quelen* have been found was of 5–6‰, and even reached 13‰, in estuaries of Rivers of the States of Paraná and Rio Grande do Sul, in southern Brazil, the same region of the country of the populations of *R. quelen* used in this study (Schiller, 2004; Loebmann and Vieira, 2006; Milani e Fontoura, 2007; Vitule, 2007). The range of habitat occurrence of *R. quelen* is thus compatible with its good capacity to regulate tissue water upon hyper-osmotic challenge, and allows us to consider the

group submitted to 5 g/L as additional controls. The group of fish submitted *in vivo* to the 5 g NaCl/L treatment displayed no difference at all with respect to freshwater controls, for all parameters evaluated, including behavioural observations. Likewise, the result of poor tissue water regulation previously observed with the other freshwater siluriform, *C. ehrhardti*, is also compatible with the habitat distribution of members of its family, Callichthyidae. The family Callichthyidae is a monophyletic group restricted to fresh water, with 177 species divided among 8 genera, occupying all the main river basins of the neotropical region (Shimabukuru-Dias et al., 2004). Phylogenetically, this family is placed distant from other groups of Siluriforms that include the family Heptapteridae of *Rhamdia quelen*, and the families Plotosidae and Ariidae, of marine catfishes (Reis, 1998; Shimabukuro-Dias et al., 2004; Nelson 2006; Froese and Pauly, 2008; Vergara et al., 2008). The relative euryhalinity of *R. quelen* is thus compatible not only with its range of distribution, but also with the phylogeny within Siluriforms.

The enzyme carbonic anhydrase (CA) of the gills of aquatic animals is involved with osmoregulation, CO_2 excretion, and acid–base regulation through the reversible reaction of CO_2 hydration, which results in hydrogen ion (H^+) and bicarbonate (HCO_3^-); CA may be soluble in the cytosol or associated to the plasma membrane (Conley and Mallatt, 1988; Randall and Brauner, 1998; Henry and Swenson, 2000; Henry et al., 2003; Georgalis et al., 2006a,b; Perry and Gilmour, 2006). In freshwater fish, CA is very relevant for osmoregulation as it supplies the branchial cell with HCO_3^- and H^+, which are respectively exchanged by Cl^- or Na^+, or even pumped out (H^+) by the vacuolar apical H^+-ATPase, thus allowing for the active absorption of salt (Conley and Mallatt, 1988; Randall and Brauner, 1998; Henry and Swenson, 2000; Kirschner, 2004; Evans et al., 2005; Perry and Gilmour, 2006; Evans, 2008). The observed activation of this enzyme in the gills of the jundiás exposed to 25 g/L of salt could hardly be associated to its role in plasma hyper-regulation, as the animal in then facing excess of external salt, with passive diffusive influx of salt through the branchial epithelia and the skin (Evans, 1993; Jobling, 1995; Reubush and Heath, 1997; Evans et al., 2005; Prodocimo et al., 2007).

Stressful situations, such as when an increase in external salinity is offered to freshwater animals, beyond their osmoregulatory capacity (here, 1 h in 25 g/L for the jundiá), evoke many metabolic disturbances. There is increase in metabolism (e.g., Martínez-Álvarez et al., 2002; Sangiao-Alvarellos et al., 2003; Soengas et al., 2007), in CO_2 production (Perry and Gilmour, 2006), in plasma cortisol (Redding and Schreck, 1983; Reubush and Heath, 1997; Mommsen et al., 1999; Tsuzuki et al., 2001; Martínez-Álvarez et al., 2002; Barreto and Volpato, 2006; Hosoya et al., 2007), and in glucose yield from glycogen degradation (Redding and Schreck, 1983; Reubush and Heath, 1997; Barcellos et al., 2001; Martínez-Álvarez et al., 2002; Gomes et al., 2003; Sangiao-Alvarellos et al., 2003; Barreto and Volpato, 2006; Gomes et al., 2006). The larger production of CO_2 ends up in metabolic acidosis (Perry and Gilmour, 2006), given the low buffering capacity of the bicarbonate system in fishes (e.g. Evans et al., 2005). Actually, the increase in blood CO_2 that results from increased metabolism is potentially enhanced by a probable reduction in branchial permeability to minimize salt entry (e.g., due to mucous production, e.g., Gomes et al., 2003; Gomes et al., 2006; Garcia et al., 2007), thus demanding a higher activity of the CA (present in red blood cells and in gills) for acid–base regulation (Henry and Swenson, 2000). Although without a direct evaluation on the acid–base balance of the jundiás, this status of metabolic acidosis can be indirectly inferred. A proportionally greater increase in plasma Cl^- than in plasma Na^+ (Truchot, 1992; Jensen et al., 1998) was observed in the jundiá submitted to increased salt. The Na^+/Cl^- ratio was of 1.21 ± 0.03 in fresh water, was not different in 5 g/L (1.18 ± 0.03) (P = 0.53, Student's t-test, compared to controls in fresh water), decreasing to 1.10 ± 0.04 in 15 g/L (P = 0.045), and decreasing further in 25 g/L, to a value below unity: 0.92 ± 0.07 (P = 0.0009). In teleosts of variable environments and latitudes, plasma Na^+ is higher than plasma Cl^- (reviewed in Freire and Prodocimo, 2007), compatible with the indication of acidosis from the relative increase in plasma Cl^- (Jensen et al., 1998). Increases in salinity and in the supply of CO_2 reportedly lead to increased CA activity (Henry and Swenson, 2000), compatible with our results. Moreover, injection of CA inhibitor (e.g. acetazolamide) leads to acid–base unbalance (Randall and Brauner, 1998; Henry and Swenson, 2000; Georgalis et al., 2006a). It should be pointed out that, as the gills of the jundiás were not perfused here, CA activity measured in the gill homogenate can at least partially reflect CA activity in the red blood cells. In particular, this association between an extreme osmotic challenge and acid–base unbalance in freshwater fishes has also been shown also in trouts (Maxime et al., 1991) or else in other aquatic animals such as the Chinese crab *Eriocheir sinensis* (Truchot, 1992).

Confirming the general pattern of homeostasis disturbance in the jundiás exposed for 1 h to 25 g/L of salt, plasma glucose increased (Laiz-Carrión et al., 2002; Martínez-Álvarez et al., 2002; Sangiao-Alvarellos et al., 2003). However, plasma cortisol remained unchanged upon all treatments. Increased salt normally results in increased plasma cortisol in FW fishes (Redding and Schreck, 1983; Reubush and Heath, 1997; Mommsen et al., 1999; Tsuzuki et al., 2001; Martínez-Álvarez et al., 2002). However, cortisol increase is strongly associated to handling stress (e.g., Barcellos et al., 2001, 2004). As basal levels of plasma cortisol for this species are ~30 ng/mL (Barcellos et al., 2001, 2004), the values reported here probably indicate handling stress irrespective of the presence of salt. Actually, depending on the type and duration of the stress, and the species, the response of increased plasma cortisol and glucose is rather variable (e.g., Hosoya et al., 2007). In addition, cortisol increase is considered a primary response to stress, followed by (secondary response) increase in blood glucose and osmoregulatory disturbance (Redding and Schreck, 1983; Reubush and Heath, 1997; Mommsen et al., 1999; Tsuzuki et al., 2001; Martínez-Álvarez et al., 2002), in agreement with our results.

Specimens of *Rhamdia quelen*, when submitted for 1 h to 25 g/L of salt (NaCl), display loss of extracellular homeostasis, with increased osmolality and ionic concentrations, raised plasma glucose, and activation of branchial carbonic anhydrase, revealing a clear stress response, albeit without increase in plasma cortisol. However, even under these circumstances of intense osmotic stress, the fish has shown remarkable capacity to regulate the water of its muscle tissue. Thus, in order to remove the parasite *I. multifiliis*, for a treatment lasting 1 h, NaCl should be used to a maximum concentration of 15 g/L. This amount of salt does not significantly affect the osmotic and acid–base homeostasis of the jundiá, as was verified using 25 g/L. This study highlights the need to investigate the effect of salt on the osmotic homeostasis of other cultured species of freshwater fishes.

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