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South American characids share very similar ionoregulatory characteristics

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A B S T R A C T

We examined ionoregulatory characteristics of four Characids from diverse locations in South America, emperor tetras (*Nematobrycon palmeri*), penguin tetras (*Thayeria boehlkei*), serpae tetras (*Hyphessobrycon eques*), and rosy tetras (*Hyphessobrycon rosaceous*). When held in 100 μmol L^{-1} Na^+ water, tetras had J_{max} values over 1100 nmol g^{-1} h^{-1}, and K_m values below 60 μmol L^{-1}. When held in 1 mmol L^{-1} Na^+ water kinetic parameters were unchanged. Low pH had no effect on Na^+ uptake (J_{in}^{Na}). At pH 3.25, Na^+ loss (J_{out}^{Na}) was stimulated 35–85% in two of the four species. To test the linkage of J_{in}^{Na} to NH_3 and H^+ extrusion we measured J_{in}^{Na} during exposure to 1 mmol L^{-1} NH_4Cl (HEA) and 100 μmol L^{-1} Acetazolemide (AZ). HEA stimulated J_{in}^{Na} of emperor tetras by 40%, but inhibited J_{in}^{Na} of penguin tetras by 50%; the two remaining species were unaffected. AZ (an inhibitor of carbonic anhydrase) inhibited J_{in}^{Na} of serpae tetras by 40%, but had no effect on the others. All tetras displayed ionoregulatory characteristics that are very similar to each other, which supports the argument that these physiological traits may be ancestral for this group and pre-date colonization of the Rio Negro. The novel finding that, J_{max} and K_m did not change after acclimation to 1 mM Na^+ water indicates that, unlike in other species examined uptake is not plastic. The HEA and AZ results, along with pH insensitivity suggest Na^+ uptake is not coupled to H^+ extrusion or NH_3 excretion and leaves the exact mechanism involved unclear.

1. Introduction

The waters of the Rio Negro in the Amazon basin are extremely dilute and acidic, with concentrations of Na^+, Cl^-, and Ca^{2+} all < 30 μmol L^{-1} and a pH of 4.85 or lower (Furch, 1984; Gaillardet et al., 1997). Such conditions pose significant challenges for ion regulation in fish, and a typical North American trout or shiner would not survive more than a few hours in such waters (McDonald, 1983). Nonetheless, the Rio Negro is a species-rich system with well over a thousand species of fish, more than in all of North American waters combined (Val and de Almeida-Val, 1995). One group of fish that has been particularly successful is the order Characiformes with at least 12 families and 1200 species (Val and de Almeida-Val, 1995). Several studies have shown that Characiform species possess a range of specializations that allow them to maintain salt balance in Rio Negro water. Perhaps chief among them is a high capacity, high affinity Na^+ transport mechanism that is insensitive to water acidity down to at least pH 3.25 (Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001; Gonzalez et al., 2002; Preest et al., 2005), which allow Characiforms to maintain very high rates of Na^+ uptake in ion-poor waters, even at very low pH levels. Such pH insensitivity is unlike virtually all other freshwater fish examined to date (Gonzalez et al., 2005).

Until recently it was generally thought that such specializations were the product of natural selection as fish colonized the harsh waters of the Rio Negro (Gonzalez et al., 2005), but recent work suggests that the origin of these traits may actually pre-date the formation of the Rio Negro (Gonzalez et al., 2017). Two Characiforms that are not native to the Rio Negro or Amazon River were found to possess the same transport characteristics, including pH insensitive uptake, as their Rio Negro relatives. One of the species, Congo tetras (*Phenacogrammus interruptus*), is from Africa, which suggests that these traits appeared early in the evolution of Characiforms before Africa and South America were even separated or the Rio Negro formed. If true, this implies that Characiform success in the Rio Negro may have resulted, not from successful adaptation to Rio Negro waters, but rather from the pre-existence of specializations that allowed them to colonize new habitats that were too harsh for less tolerant species. Still, these intriguing implications are based on examination of just a handful of species from the species-rich order Characiformes. Consequently, the first goal of this study was to increase the number of species examined by evaluating 4 additional species, three of which are not native to the Rio Negro. Evaluation involved estimating kinetic parameters and pH sensitivity of Na^+ transport in these species, as well as assessing sensitivity to a couple of water treatments, high external ammonia and acetazolemide, that have been used previously to gain some insight into the nature of ion transport mechanisms (Preest et al., 2005; Gilmour and Perry, 2009; Wood et al.,

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2014; Gonzalez et al., 2017).

One possible criticism of the evolutionary scenario just described is that the high affinity/high capacity transporters may reflect acclimation to ion-poor waters. In three species examined this way, Na^+ transport capacity and transporter affinity for Na^+, have been shown to acclimate to high Na^+ waters (McDonald and Rogano, 1986; Gonzalez et al., 2002; Boisen et al., 2003). In these species, transporters of fish held in higher Na^+ concentrations have lower affinity and capacity than fish held in low Na^+ concentrations. If such were the case for characiforms, then this would undermine the utility of these traits in an evolutionary context. To assess this, our second goal was to estimate transport affinity and capacity in fish held in water with low Na^+ and high Na^+ concentrations.

2. Materials and methods

2.1. Experimental animals

We acquired four species of fish from the family Characidae from a local tropical fish supplier, Pet Kingdom. Emperor tetras (n = 24; 0.327 ± 0.025 g; *Nematobrycon palmeri*) are native to the upper reaches of the Rio Negro and have been previously shown to be very tolerant of low pH (Dunson et al., 1977). Penguin tetras (n = 30; 0.512 ± 0.024 g; *Thayeria boehlkei*), and Serpae tetras (n = 30; 1.039 ± 0.053 g; *Hyphessobrycon eques*) are native to streams in the central Amazon basin, but not the Rio Negro. Rosy tetras (n = 30; 1.324 ± 0.101 g; *Hyphessobrycon rosaceous*) are native to streams and rivers or coastal Guyana and Surinam. All fish were held in 60–80 L aquaria filled with de-ionized water to which salts had been added to reach the desired concentrations (100 μmol L^{-1} both NaCl and $CaCl_2$, pH = 7–8, adjusted with KOH). Water pH was monitored daily and adjusted as needed. Water was changed every two weeks to ensure nominal concentrations were maintained. Total fish mass per aquarium was typically 10–15 g or less. Water temperature was 25–27 °C (all tests were performed at this temperature range). The fish were fed flake food *ad libitum* until at least 24 h before the start of experiments.

2.2. Experimental protocol

We measured net Na^+ flux (J_{net}^{Na}) and Na^+ influx (J_{in}^{Na}), and calculated Na^+ efflux (J_{out}^{Na}) for fish under various conditions. To make measurements fish were weighed and placed into individual 65-mL chambers connected to a 60-L re-circulating system that was filled with water with identical composition and temperature as water in the holding tanks. Fish were allowed to recover for at least 18 h. To begin a measurement period flow was stopped to all containers, radioisotope $^{22}NaCl$ was added to each chamber (5.5 kBq per chamber), and, after a 5 min mixing period, a 6-mL water sample was removed. One hour later another water sample was removed and flow was restored. One milliliter from each water sample was mixed with 5-mL of scintillation cocktail and assayed for ^{22}Na activity with a liquid scintillation counter. The remaining 5-mL of each sample was assayed for Na^+ concentration with an atomic absorption spectrophotometer. J_{net}^{Na} was calculated from the change in the Na^+ concentration of the chamber water over the one-hour using the following equation:

$$J_{net}^{Na} = ([Na]_i - [Na]_f)\, V\, (M\, t)^{-1}$$

where $[Na]_i$ and $[Na]_f$ are the chamber Na^+ concentrations at the beginning and end of the flux period, respectively, V is the bath volume in liters, M is the mass of the fish in grams, and t is the duration of the flux period in hours.

For all tests, except Na^+ uptake kinetic measurements, J_{in}^{Na} was calculated from the disappearance of isotope from the water and the average Na^+ concentration of the water during the flux period (Gonzalez and Dunson, 1987) with the following equation:

$$J_{in}^{Na} = (\ln \underline{Q}_{out0} - \ln \underline{Q}_{out1})\underline{Q}_{out}\, (\underline{M}^*\, \underline{t})^{-1}$$

where $\underline{Q}_{out0}$ and $\underline{Q}_{out1}$ are the total counts per minute in the flux chambers at the beginning and end of the flux period, respectively, $\underline{Q}_{out}$ is the average amount of Na^+ in the flux chamber during the flux period, $\underline{M}$ is the mass of the fish in grams, and $\underline{t}$ is the time in hours. J_{out}^{Na} was calculated for each fish from the difference between J_{net}^{Na} and J_{in}^{Na}.

Na^+ uptake kinetics measurements involved six consecutive flux periods (see below) so we employed a modified equation for J_{in}^{Na} that corrects for backflux due to accumulation of isotope over the six flux periods (Wood, 1988). For the calculations we used the following equation:

$$J_{in}^{Na} = ([R]_i - [R]_f)V_{ext} - SA_{int}\,([Na]_i - [Na]_f)/(SA_{int} - SA_{ext})M$$

where $[R]_i$ and $[R]_f$ are initial and final radioactivities (in counts min^{-1} mL^{-1}), SA_{int} and SA_{ext} are the mean internal and external specific activities (in counts min^{-1} μmol $^{-1}$ Na) over the flux period. For calculation of SA_{int}, a value of 300 mL kg^{-1} was used for internal distribution volume and 50 μmol g^{-1} was used for the total exchangeable internal pool of Na^+ (Wood, 1988). $[Na]_i$ and $[Na]_f$ are the initial and final water Na^+ concentrations (in μmol mL^{-1}), V_{ext} is the volume of the flux chamber (in mL), and M is mass in kg.

2.3. Experimental series

Na^+ uptake kinetics – Na^+ uptake kinetic parameters were estimated in fish held in 100 μmol L^{-1} Na^+ water for several months and after 1 month in 1 mmol L^{-1} Na^+ water. The lower concentration is the standard concentration we have used previously for ion-poor waters (Gonzalez et al., 2017), the higher concentration is in the range used in previous studies to represent ion-rich water (Boisen et al., 2003; McDonald and Rogano, 1986). Sodium uptake was measured during exposure to 6 sequentially increasing water sodium concentrations starting at 10 μmol L^{-1} and going up to 320 μmol L^{-1}. To make measurements, fish were placed in the chambers connected to the re-circulating system and allowed to recover overnight in water identical to that in the holding tanks. To begin the experiment, we stopped flow to individual chambers, drained the system, rinsed it, refilled it with water with a calcium concentration of 100 μmol L^{-1} and pH 7–8 (adjusted with KOH), as in holding water, and added NaCl to reach the first target concentrations. After a 15-min exposure to the first (lowest) Na^+ concentration, flow was stopped, isotope was added and a 1-h flux period was initiated. While the flux period was occurring an aliquot of 1 M NaCl stock solution was added to the re-circulating system reservoir to raise the Na^+ concentration of the water to the next target concentration. Upon completion of the flux period and restoration of flow fish began exposure to the next, higher Na^+ concentration. After a 15-min another flux period was begun. This process was repeated until fish had experienced all 6 Na^+ concentrations. At the two highest Na^+ concentrations the amount of isotope used was doubled to maintain a high specific activity. The Michaelis-Menten constant (K_m), a measure of the transport mechanism's affinity for Na^+, and the maximum transport capacity (J_{max}) were estimated with non-linear regression, using the standard Michaelis-Menten equation for enzyme saturation $J_{in}^{Na} = (J_{max}\ [Na^+]) / (K_m + [Na^+])$ as the model (Gonzalez et al., 1997; Boisen et al., 2003).

Unidirectional Na^+ fluxes at low pH – To assess the acute responses of ion regulation to low pH, we measured unidirectional Na^+ fluxes during serial exposure to pH 7.5 (control), 4.0, and 3.25. pH 4.0 was chosen because it is the pH level where uptake is typically fully inhibited in pH-sensitive species (McWilliams, 1980; McDonald et al., 1980; Freda and McDonald, 1988), and pH 3.25 was chosen because previous work has shown that characids can tolerate this pH and still transport ions (Gonzalez and Preest, 1999). A 1-h flux at pH 7.5 was performed first. While that flux was occurring, the pH of the water in