Spring 5-28-2017

Investigation into the Mechanism of Ion Transport in Rio Negro Characiformes

Vineza D. Reduta
University of San Diego

Investigation into the Mechanism of Ion Transport in Rio Negro Characiformes

_____

A Thesis

Presented to

The Faculty and the Honors Program

Of the University of San Diego

_____

By

Vineza Reduta

Biology Department

2017

Honors Thesis Approval Page

Student Name: Vineza Reduta

Title of Thesis: Investigation into the Mechanism of Ion Transport in Rio Negro Characiformes

Accepted by the Honors Program and faculty of the Department of Biology, University of San Diego, in partial fulfillment of the requirements for the Degree of Bachelor of Arts.

FACULTY APPROVAL

| _____ | _____ | _____ |
| Faculty Project Advisor (Print) | Signature | Date |

| James Gump | _____ | _____ |
| Honors Program Director | Signature | Date |

Abstract

Four fish species within the Characiform Order [(Rosy tetras (*Hyphessobrycon rosaceus*), Serpae tetras (*Hyphessobrycon eques*), Emperor tetras (*Nematobrycon palmeri*) and Penguin tetras (*Thayeria obliqua*)] are native to the ion-poor, acidic waters of the Rio Negro. In this paper, we focus on Wood's revised Na^+/NH_4^+ exchange model to gain better insight into the mechanisms of ion transport within the Characids. We exposed each species to two experimental treatments: (i) 1mM ammonia (high external ammonia [HEA]), to test coupling of Na^+ uptake and ammonia excretion, and (ii) 100μM Acetazolamide (carbonic anhydrase inhibitor), to test coupling of Na^+ uptake and H^+ excretion. With the HEA treatment, three out of the four species resulted in neither a stimulation nor inhibition of Na^+ uptake, indicating no loose coupling involved between NH_4^+ excretion and Na^+ uptake. As for the Acetazolamide exposure, three out of the four species showed no significant difference in Na^+ uptake, showing that Na^+ uptake is not dependent on the protons provided from carbonic anhydrase. Thus, Wood's Na^+/ NH_4^+ exchange model is unsupported in these Characiformes.

Introduction

One of the largest tributaries of the Amazon River is the Rio Negro, which translates to Black River in Spanish. The Rio Negro has dark, tea colored waters due to the abundance of Dissolved Organic Carbon (DOC) that derive from decaying plant material. It also has extremely dilute ion concentrations (μmol L^{-1}: Na^+= 16.5 $\pm$ 5.3, K^+= 8.2 $\pm$ 2.7, Ca^{2+}= 5.3 $\pm$ 1.6, Mg^{2+}= 4.7$\pm$ 1.4, and Cl^-= 47.9 $\pm$ 19.7), which leads to a low buffering capacity (Furch, 1984). Consequently, the waters are acidified by organic acids contained in the DOC (pH$\leq$ 4.5; Furch, 1984). The low ion concentrations and high acidity, make its waters a harsh environment for fish

as they experience challenges in maintaining ion regulation (Gonzalez et al., 1997). Despite these harsh environmental conditions, these waters are home to more than 1,000 different species (Val and Almeida, 1995).

In order to understand survival of these fish in the Rio Negro, it is important to understand basic ion regulation and physiology of freshwater fish. After much experimentation within the past 50 years, scientists have obtained data supporting that the branchial epithelium (located in the fish gill and separates the interior cells from the external environment) is involved in acid/base regulation and nitrogenous waste excretion. Additionally, it has been established that it is also a primary site of ion regulation (Evans et al., 2005). In typical freshwater environments, fish are exposed to a hypotonic environment, in which they have higher salt concentrations in their blood (around 150 mM Na^+; 130 mM Cl^-) than that of their surrounding water ($\leq$ 1 mM Na^+, Cl^-) (Evans, 1979). This results in a diffusion gradient across the fish's gills, which drives diffusive loss, or efflux. Thus, to maintain equilibrium, fish must actively take up salts from the water and this also occurs at the gills (Evans et al. 2005).

To describe how efflux occurs within fish, scientists have proposed a mechanism regarding how Ca^{2+} ions particularly affect Na^+ efflux. This mechanism involves gill permeability influenced by $[Ca^{2+}]$ of the surrounding water, in which Na^+ ions exit the junction between two epithelial cells into the external environment. Under normal conditions when there is a high $[Ca^{2+}]$ in the external environment, Ca^{2+} ions bind to junction proteins between the cells. When these ions are bound, this causes the proteins to decrease the separation between the two epithelial cells and decreases permeability (McDonald et al, 1980; McDonald & Wood, 1981; McDonald, 1983). Thus, less Na^+ ions are able to exit the junctions resulting in low Na^+

efflux. However, this efflux mechanism is still uncertain and not fully understood (McDonald 1983).

To better understand how fish can manage a state of equilibrium once ions are lost from their body, it is best to dive into how fish are then able to gain back ions into their internal environment via ionic transport. Throughout the past decades, scientists have proposed three different models as to how these fish can actively transport salts into their bodies from the external environment. These three mechanisms are currently not fully understood and many scientists are still debating on which one is the correct model for the fish (Lin and Randall 1991, 1993; Potts 1994). The models all involve the movement of protons exiting into the external environment for Na^+ uptake to occur.

The first transport model involves a Na^+/K^+ ATPase and a Na^+/H^+ exchanger (Figure 1; Maetz and Garcia-Romeau, 1964). The Na^+/K^+ ATPase is embedded within the cell's basolateral membrane that has direct contact with internal fluids of the fish. This ATPase is an enzyme that acts as a pump moving Na^+ ions out of the cell and K^+ ions into the cell. This effect lowers the $[Na^+]$ inside the cell. On the apical membrane of the fish, a Na^+/H^+ exchanger is present. This exchanger moves protons out of the cell into the external environment while moving Na^+ ions from the external environment into the cell at the same time. Overall, Na^+ ions are moving against the concentration gradient (Maetz and Garcia-Romeau, 1964). So, this model is an active transport.

The second model involves basolateral Na^+/K^+ ATPase, but with an ATPase H^+ pump and a Na^+ channel on the apical side (Figure 2). The Na^+/K^+ ATPase has the same function as that of first model stated above by driving Na^+ ions out into the bloodstream and replacing it with K^+ ions into the cell. H^+ ATPase drives protons out into the external environment, resulting

in an overall negative potential within the cell relative to the water. This negative potential attracts the Na^+ ions from the external environment into the cell by moving against its concentration gradient via Na^+ channels (Efrenfeld et al, 1985; Avella and Bornancin 1989; Sullivan et al, 1995). Since more Na^+ ions are present in the cell, this continues the cycle of the active transport with the Na^+/K^+ ATPase.

The final model is based upon a mechanism first presented by August Krogh (1938), in which he believed that Na^+ uptake might be coupled to $NH4^+$ excretion. Then in 2014, Wood proposed a revised model of Krogh's mechanism that involves two different sources of protons. The first source of protons is through the Na^+/NH_4^+ exchanger, in which NH_4^+ replaces K^+ in Na^+/K^+ ATPase. This exchanger is embedded within the cell's membrane and makes direct contact between the bloodstream and the fish's cells (Figure 3). This port drives Na^+ ions from the cell into the bloodstream, and in return, allows NH_4^+ ions to move from the bloodstream into the cell. Then once inside the cell, the NH_4^+ separates into H^+ and NH_3 (Wood et. al, 2014). NH_3 moves down its concentration gradient into the external environment through a transporter and H^+ is supplied to Na^+/H^+ exchanger. The NH_3 combines with the protons in the external environment and forms NH_4^+, which keeps external $[H^+]$ and $[NH_3]$ low. As for the second source of protons, this is provided through a carbonic anhydrase (Figure 4). This is an enzyme that converts CO_2 and H_2O into HCO_3^- and H^+, supplying H^+ for Na^+/NH_4^+ exchange (Wood et al, 2014).

Dilute, low pH waters pose challenges for ion transport as uptake is concentration dependent. With dilute waters, a lower concentration of Ca^{2+} ions in the external environment result in less Ca^{2+} ions binding to the junction proteins, resulting in an increase in Na^+ efflux (McDonald et al, 1980; McDonald & Wood, 1981; McDonald, 1983). Acidic waters also

influence the junction proteins as well. When freshwater fish are exposed to low pH, the higher concentration of H^+ ions in the water tends to replace the Ca^{2+} ions that typically bind to the junction proteins between the epithelial cells. This results in an increase in permeability between the epithelial cells because the H^+ ions are now bound directly to the junction proteins instead of the Ca^{2+} ions, resulting in a high rate of Na^+ efflux to occur (Gonzalez et al., 2002). Thus, dilute, low pH waters result in an overall loss of ions within freshwater fish, ranging anywhere between -3000 to -9800 nmol g^{-1} h^{-1}. However, if more than 50% of ions are lost from the body, this may lead to mortality (Gonzalez and Dunson, 1987; McDonald et al., 1991).

Our research focuses on studying the mechanisms of ion transport in Rio Negro fish. Particularly, native Rio Negro Characid studies have shown that Na^+ transport has a high affinity for Na^+ (low K_m) and a high transport capacity (high J_{max}) mechanism (Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001). To clarify, K_m is the substrate concentration at half the maximum velocity and J_{max} is the transport capacity. A high affinity for Na^+ and a high transport capacity indicate these Characids only need a small presence of Na^+ ions in the external environment in order for Na^+ to bind to the transporter. This allows the Characids to have a high rate of uptake even in very dilute waters. Also, uptake is insensitive to pH, which enables them to maintain Na^+ uptake at low pH levels (Gonzalez and Wilson, 2001). These specializations indicate their high tolerance in these harsh environmental conditions and call into question the mechanism of Na^+ transport in these fish.

In this paper, we examined Na^+ transport in four different species of Characids [Rosy tetras (*Hyphessobrycon rosaceus),* Emperor tetras (*Nematobrycon palmeri*), Penguin tetras (*Thayeria boehlkei*), Serpae tetras (*Hyphessobrycon eques*] native to the Rio Negro to evaluate the mechanism of uptake. We particularly focused on Wood's Na^+/NH_4^+ exchange model, which

suggests a loose coupling of Na^+ uptake with NH_4^+ excretion, in which the inhibited NH_4^+ excretion could stimulate Na^+ uptake by providing more H^+ for Na^+ exchange. Thus, we exposed these fish to two different treatments: 1) high external ammonia, which tests the coupling of Na^+ uptake and ammonia excretion and 2) acetazolamide, which is known as a carbonic anhydrase inhibitor, to test the coupling of Na^+ uptake and H^+ excretion.

Materials and methods

Experimental animals

      Rosy tetras (*Hyphessobrycon rosaceus),* Emperor tetras (*Nematobrycon palmeri*), Penguin tetras (*Thayeria boehlkei*), Serpae tetras (*Hyphessobrycon eques*) were commercially obtained from San Diego's Pet Kingdom and held in aquaria with deionized water and salts to reach a final solute concentration of $100\mu M$ Na^+ and Ca^{2+} and pH 7-8. Water temperature was 26^oC. The fish were fed Tetramin flake food ad lib, until at least 24 hours before the start of experiments.

Experimental protocol

      Fish were weighed and placed into individual plastic flux chambers connected to a 100L recirculating system that was filled with water identical to the water in the holding tanks. Once in the chambers, fish were left to acclimate to overnight to allow for recovery from stress due to handling. At the beginning of a measurement period, flow was stopped to all containers, $150\mu L$ of radioisotope $^{22}NaCl$ was added to each chamber, and after a five-minute mixing period, a 6mL water sample was removed. One hour later, another water sample was removed, and flow was restored. As for the experimental flux, the same procedure stated above was performed;

however, an experimental variable was introduced in attempts to alter the mechanisms of ion transport.

For data analysis, 1mL from each water sample was mixed with 5mL of scintillation cocktail and assayed for ^{22}Na with a liquid scintillation counter. The remaining 5mL of each sample was assayed for Na^+ concentration with an atomic absorption spectrophotometer. Na^+ influx was calculated from the disappearance of isotope from the water and the average Na^+ concentration of the water during the flux period using the equation from Gonzalez and Dunson (1987): Na^+ influx= $(\ln Q_{out0} - \ln Q_{out1})/Q_{out}/(M \times t)$, where Q_{out0} and Q_{out1} are the total counts per minute in the flux chambers at the beginning and end of the flux period, respectively; Q_{out} is the average amount of Na^+ in the flux bath during the flux period; M is the mass of the fish in grams; and t is the time in hours.

Experimental Series

High External Ammonia Exposure

To test the loose coupling between Na^+ uptake and ammonia excretion, we measured J_{in}^{Na} during acute exposure to 1mmol L^{-1} NH_3Cl (HEA). A control flux was performed in ammonia-free water, followed by the test period. For the test flux, an aliquot of concentrated NH_3Cl was added to each chamber after flow was stopped, and fish were exposed for 15 min before the start of the flux measurement period.

Acetazolamide Exposure

To test if Na^+ uptake was dependent on the protons provided from carbonic anhydrase, we measured J_{in}^{Na} during acute exposure to 100μM Acetazolamide. A control flux was

performed in ammonia-free water, followed by the test period. For the test flux, an aliquot of

Acetazolamide was added to each chamber after flow was stopped and fish were exposed for 15

min before the start of the flux measurement period.

Results

High External Ammonia

Na$^+$ uptake was not inhibited in Rosy and Serpae tetras (Figure 1). On the other hand,

there was a 27% increase in uptake in the Emperor tetras ($p=0.03$), while the Penguin tetras

exhibited an approximate 50% decrease in uptake ($p=0.001$; Figure 1).

Acetazolamide

The Penguin, Rosy, and Emperor tetras displayed no significant difference in Na$^+$ uptake

(Figure 2). However, the Serpae tetras averaged around a 42% decrease in uptake ($p=0.003$;

Figure 2).

Discussion

High External Ammonia

Overall, Na$^+$ uptake was not inhibited in three of the four species (Rosy, Serpae,

Emperors tetras) tested. Therefore, there is strong evidence that there is no loose coupling

between Na$^+$ uptake and NH$_4^+$ excretion in Characid fish. If Wood's model was correct, high

external ammonia would result in a decrease in Na$^+$ uptake. Thus, this indicates that the

Na$^+$/NH$_4^+$ model proposed by Wood is unsupported within Characiformes of the Rio Negro.

The lack of inhibition shown in the Rosy and Serpae tetras suggests that ammonia excretion is not coupled with Na^+ uptake. This is similar to the results found in the Black Neon tetras as there was no significant difference in uptake (Gonzalez et al., 2016).

As for the Emperor tetras, the slight stimulation in Na^+ uptake correlates to that of a previous study done with the Cardinal tetras when exposed to high external ammonia (Wood et al., 2014). Prior to the experiment with the Cardinal tetras, Wood argued that the presence of high external ammonia would lead to a decrease in Na^+ uptake due to a cascade in disruptions in the gradients and an overall decrease in protons within the cell. However, his findings of the stimulation of uptake in the Cardinal tetras led him to revise his model. He argued that exposure to high external ammonia within this species would cause an accumulation of NH_4^+ in the cell, which would ultimately result in an increase in Na^+ uptake. Similarly, Gonzalez (2016) also observed a stimulation in uptake in the Congo tetras. On the other hand, with further experimentation in which he exposed the fish to low pH waters (≤ 3.5), Na^+ uptake was not inhibited. Thus, this suggests that Na^+ uptake is not coupled with proton excretion, refuting Wood's Na^+/NH_4^+ model.

Lastly, the 50% decrease in uptake exhibited in the Penguin tetras indicates that there may be a loose coupling involved between exposure to high external ammonia and Na^+ uptake.

Acetazolamide

The no inhibition shown in the three of the four species (Rosy, Penguin, and Emperor tetras) studied suggests that Na^+ uptake is not dependent on the protons provided from the carbonic anhydrase. They may be obtaining the H^+ ions from another proton source that has yet to be discovered. As for the Serpae tetras, the 42% decrease in uptake demonstrates that this

species may be dependent on the carbonic anhydrase for protons in order for uptake to occur. This result is similar to that of the Cardinal tetras, as a 50% inhibition in uptake was observed (Wood et al., 2014).

However, overall three of the four species (Rosy, Penguin, and Emperor tetras) studied displayed no significant change in Na^+ uptake. This displays stronger support that Na^+ uptake is not dependent on H^+ ions from carbonic anhydrase, further refuting Wood's Na^+/NH_4^+ model.

Future Experiments

These two experiments demonstrate that Wood's Na^+/NH_4^+ model is unsupported within the Characiform Order. We are still unsure ourselves about the correct ion transport mechanism. But, we do believe there may be a novel mechanism that is still yet to be discovered.

As for future directions, it would be interesting to further test the other two proposed mechanisms of ion transport: (i) Na^+/H^+ Exchanger and (ii) H^+ ATPase pump/Na^+ channel. This can be done by exposing the fish to certain pharmaceutical drugs in attempts to alter the ion transport mechanisms. Several drugs, such as Bafilomycin (H^+ ATPase pump blocker) and Dimethoxyamphetamine (DMA; NHE blocker), should decrease Na^+ uptake if the ion transport models are correct. However, with past studies in native Rio Negro fish, these drugs had no effect on Na^+ transport. Additionally, another way to alter the mechanisms of ion transport is to expose the fish to various metals. Silver seems to have a consistent effect in inhibiting Na^+ uptake because its strong charges bind readily to any protein inside the cell, having a non-specific inhibition (Wood et al., 2014). It would be interesting to expose the fish to either copper or cadmium as they are thought to block proteins located on the surface of the cell (Matsuo et al., 2005).

Also, exploring more Characiform species would be beneficial in discovering the overall consensus of ion transport within these fish. The Characiform Order has 18 families, with well-over 2,000 species. Our study focused on only four species that have not been tested before. Thus, testing more species within this Order will provide a better insight on how these fish can tolerate the harsh conditions of the Rio Negro.

Last but not least, testing other native Rio Negro Orders would also be another approach in studying ion transport. For instance, the Cichlid Order is known to have low affinity and low capacity ion transporters (Gonzalez and Preest, 1999; Gonzalez and Wilson, 2001) in contrast to the Characids, which have a high affinity and high capacity.

References

Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport
through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J. Exp. Biol.
142:155-175.

Efrenfeld, J., Garcia-Romeau, F. and Harvey, B.J. (1985). Electrogenic active proton pump
in *Rana esculenta* skin and its role in sodium transport. J. Physiol. Lond. **359**: 331-355.

Evans, D.H. (1979). Fish. Comparative physiology of osmoregulation in animals. Vol. 1.
Academic Press, London. Pp. 305-390 in G.M. O. Maloiy, ed.

Evans, D. H., Piermarini, P.M. and Choe, K. P. (2005). The multifunctional fish gill:
dominant site of gas exchange, osmoregulation, acid-Bas Regulation, and excretion of
nitrogenous waste. Physiol. Rev. **85**:97-177.

Furch K. (1984). Water chemistry of the Amazon basin: the distribution of chemical elements
among freshwaters. Pp. 167-199 in H. Sioli, ed. The Amazon: Limnology and landscape
ecology of a mighty tropical river and its basin. Junk, Dordrecht.

Gonzalez, R.J., Dalton, V.M. and Patrick, M.L. (1997). Ion regulation in ion-poor, acidic
water by the blackskirt tetra (*Gymnocorymbus ternetzi*), a fish native to the Amazon
River. Physiol. Zool. **70**: 428-435.

Gonzalez, R.J., and Dunson, W.A. (1987). Adaptations of sodium balance to low pH in a
sunfish (*Enneacanthus obesus*) from naturally acidic waters. J. Comp. Physiol. **157B**:
555-566.

Gonzalez, R.J. & Preest, M. (1999). Mechanisms for exceptional tolerance of ion-poor, acidic
waters in the neon tetra (*Paracheirodon innesi*). Physiol. Zool. **72**: 156-163.

Gonzalez, R.J., Wilson, R.W., Wood, C.M., Patrick, M.L. and Val, A.L. (2002). Diverse strategies for ion regulation in fish collected from the ion-poor, acidic Rio Negro. Physiol. Biochem. Zool. **75**: 37-47.

Gonzalez, R.J. and Wilson, R.W. (2001). Patterns of ion regulation in acidophilic fish native to the ion-poor, acidic Rio Negro. J. Fish Biol. **58**: 1680-1690.

Gonzalez, R.J., Jones, S.L. and Nguyen, T.V. (2016). Ionregulatory Characteristics of Non-Rio Negro Characiforms and Cichlids. J. Fish Biol.

Krogh, A. (1938). The active absorption of ions in some freshwater animals. Z. Vergl. Physiol. **25**:335–350.

Lin, H. and Randall, D.J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. J. Exp. Biol. **161**: 119-134.

Lin, H. and Randall, D.J. (1993). Proton ATPase activity in the crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol. **180**: 163-174.

Maetz, J. and Garcia-Romeau, F. (1964). The mechanism of sodium and chloride uptake by the gills of a freshwater fish, *Caracssius auratus*. II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. J. Gen. Physiol. **47**: 1209-1227.

Matsuo, A.Y., Wood, C.M. and Val, A.L. (2005). Effects of copper and cadmium on ion transport and gill metal binding in the Amazonian teleost tambaqui (*Colossoma macropomium*) in extremely soft water. Aquat. Toxicol. **74**: 351-364.

McDonald, D.G., Freeda, J., Cavdek, V. , Gonzalez, R. and Zia, S. (1991). Interspecific differences in gill morphology of freshwater fish in relation to tolerance of low pH environments. Physiol. Zool. **64**: 124-144.

McDonald, D.G., Hobe, H. and Wood, C.M. (1980). The influence of calcium on the physiological responses of the rainbow trout, *Salmo gairdneri*, to low environmental pH. J. Exp. Biol. **88**:109-131.

McDonald, D.G., Walker, R.L. and Wilkes, **R.L.K.** (1983). The interaction of calcium and low pH on the physiology of the rainbow trout *Salmo gairdneri*. II. Branchial ionregulatory mechanisms. J. Exp. Biol. **102**: 141-155.

McDonald, D.G. and Wood, C.M. (1981). Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. J. Exp. Biol. **93**: 101-118.

Potts, W.T.W. (1994). Kinetics of sodium uptake in freshwater animals: a comparison of ion-exchange and proton pump hypotheses. Am. J. Physiol. **266**: R315-R320.

Sullivan, G.V., Fryer, J.N. and Perry, S.F. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. J. Exp. Biol. **198**: 2619-2629.

Val, A.L. and Almeida-Val, V.M.F. (1995). Fishes of the Amazon and their environment. Physiological and biochemical features. Springer, Heidelberg.

Wood, C.M, Robertson, L.M., Johannsson, O.E. and Val, A.L. (2014). Mechanisms of Na^+ uptake, ammonia excretion, and their potential linkage in native Rio Negro tetras (*Paracheirodon axelrodi, Hemigrammus rhodostomus, and Moenkhausia diktyota)*. J. Comp. Physiol. **184**: 877-890.

H⁺   Na⁺

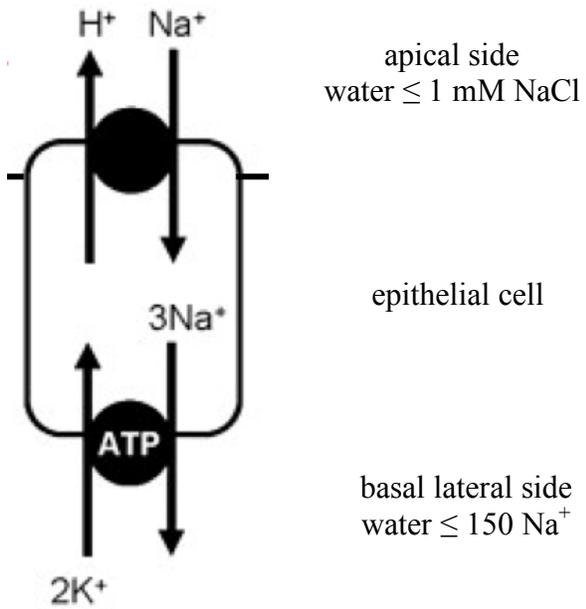apical side
water $\leq$ 1 mM NaCl

epithelial cell

3Na⁺

ATP

basal lateral side
water $\leq$ 150 Na^+

2K⁺

Figure 1. Na^+/K^+ ATPase and a Na^+/H^+ exchanger model of ion transport (Maetz and Garcia-Romeau, 1964).
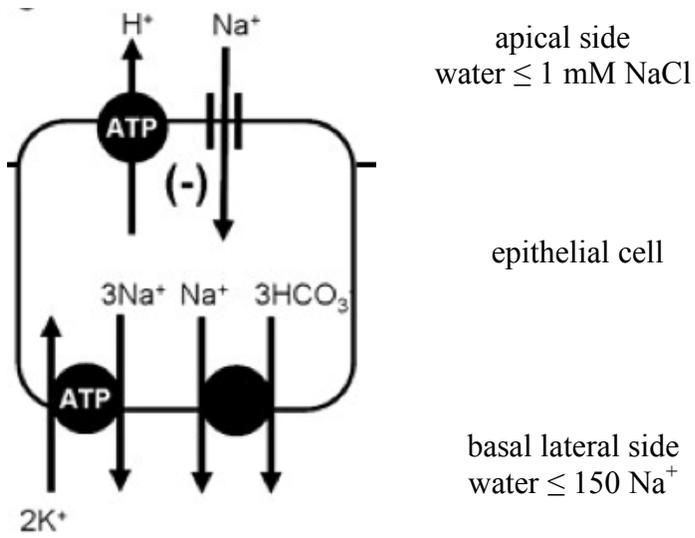
Figure 2: H$^+$ ATPase pump and Na$^+$ channel model of ion transport (Efrenfeld et al, 1985; Avella and Bornancin 1989; Sullivan et al, 1995).
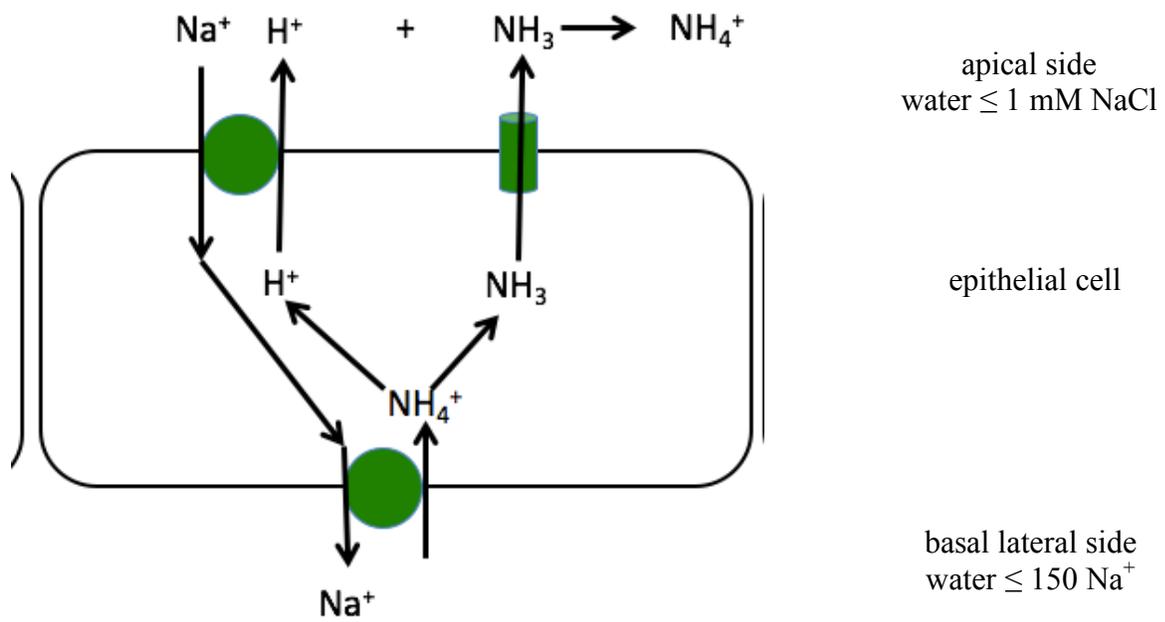
Figure 3: Wood's revised model (2014) of a loose coupling between ammonia excretion and Na^+ uptake.
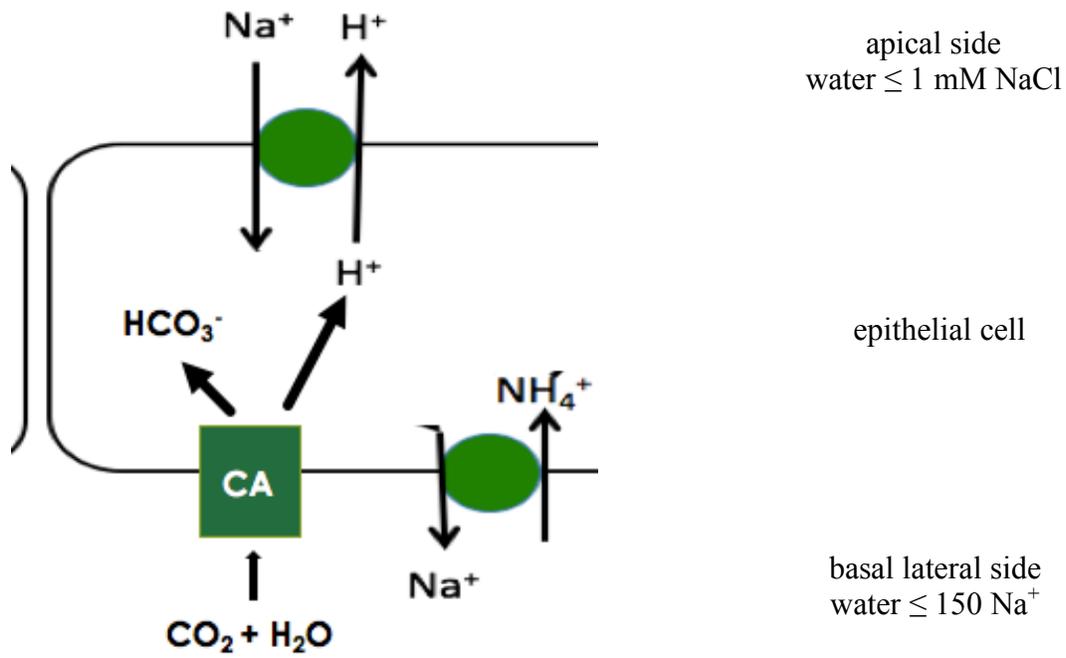
Figure 4: Wood's revised model (2014) of a second source of protons for Na^+ uptake provided from carbonic anhydrase.
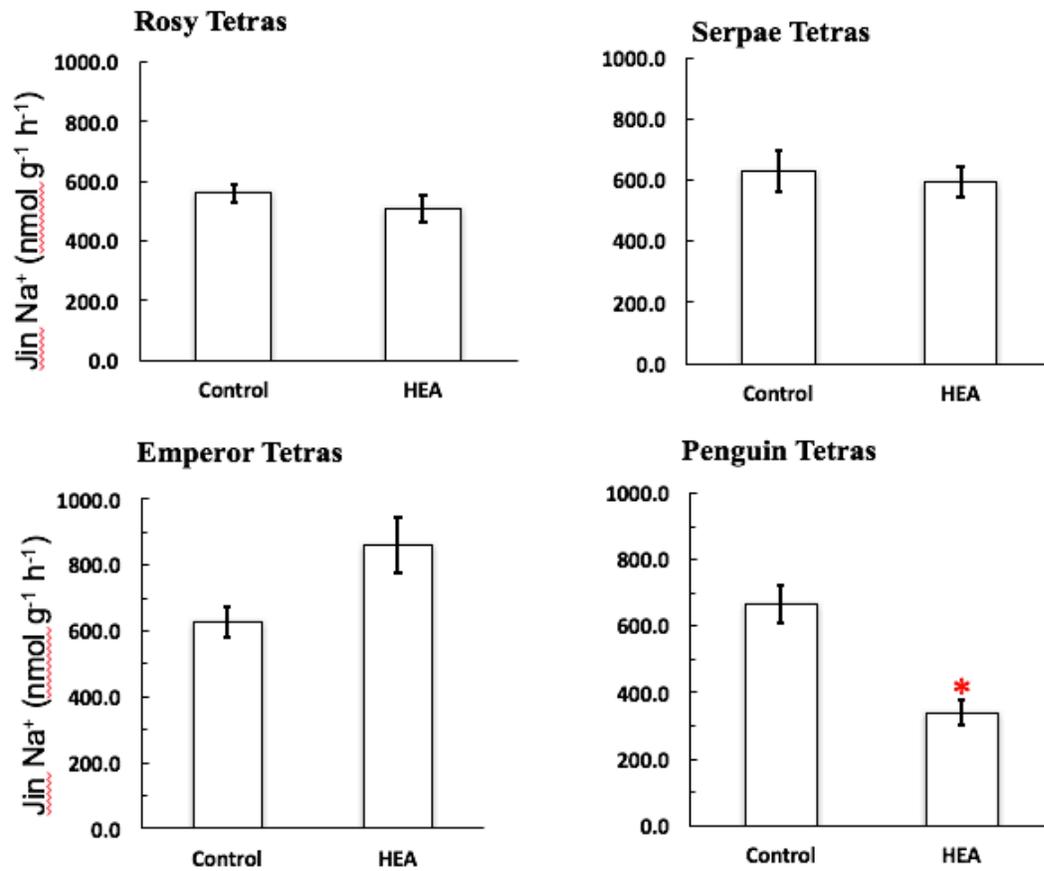
Figure 5. Effects of High External Ammonia (HEA) on Na$^+$ influx (J_{in}^{Na}) in Rosy, Serpae, Emperor and Penguin Tetras. Mean $\pm$ SE (n=6). Asterisks indicate significant differences from control fluxes.
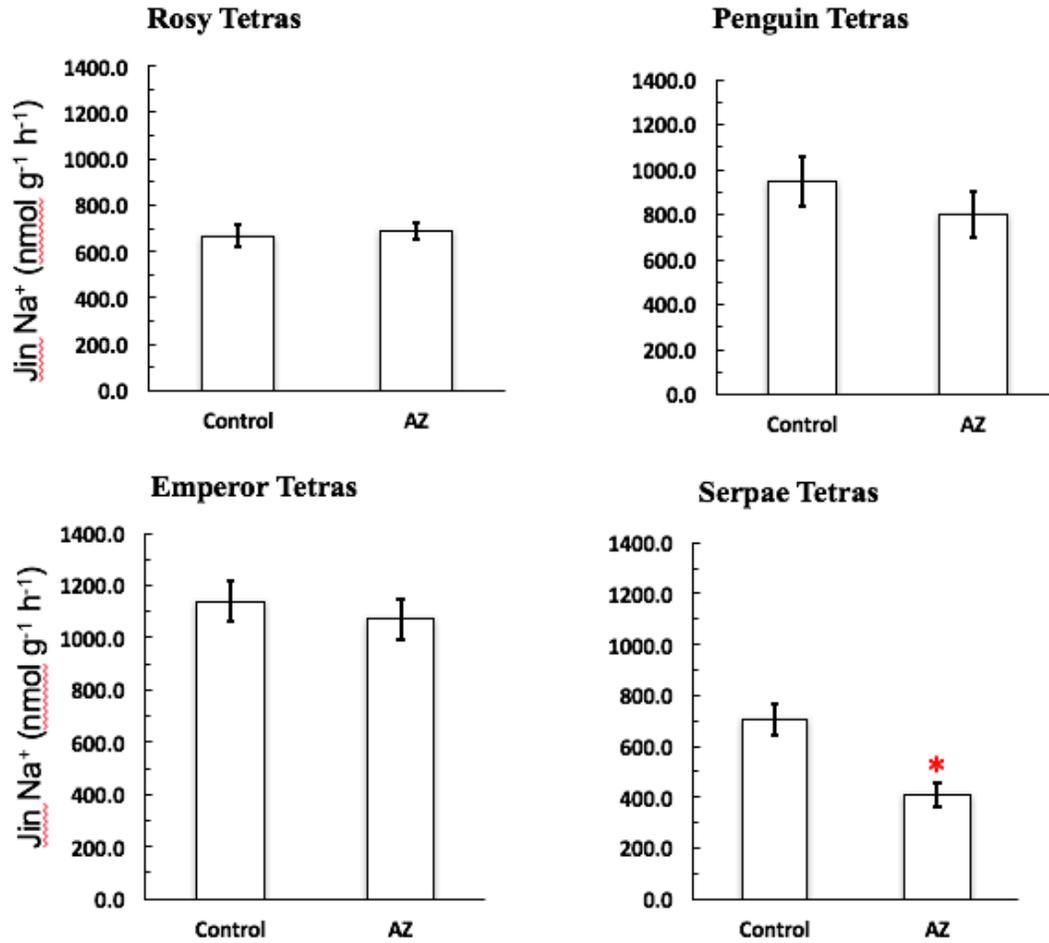
Figure 6. Effects of Acetazolamide (AZ) on Na+ influx (J_{in}^{Na}) in Rosy, Penguin, Emperor and Serpae Tetras. Mean + SE (n=6). Asterisks indicate significant differences from control fluxes.