Short communication

Effect of water pH and calcium on ion balance in five fish species of the Mekong Delta

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ARTICLE INFO

ABSTRACT

Acidic freshwater habitats disrupt ion-homeostasis in fishes, yet the often acidic waters of the Mekong host the second highest diversity of freshwater fish in the world. To investigate how five Mekong fish species tolerate water acidity, we measured: time to loss of equilibrium (LOE) at sustained (4 days) low pH (3.5) and net ion flux in acute low pH (3.5 and 3) in *Chitala ornata*, *Pangasianodon hypophthalmus*, *Osphronemus goramy*, *Trichogaster pectoralis*, and *Monopterus albus*. Our sustained low pH exposures revealed that *C. ornata* was least tolerant, *P. hypophthalmus* and *M. albus* were moderately tolerant, and *O. goramy* and *T. pectoralis* were highly tolerant to low pH. In general, net ion loss in acute low pH exposures was greatest in species with the shortest time to LOE in the sustained low pH exposure. We also explored how low water $[Ca^{2+}]$ (relative to current Mekong levels) affected ion flux at low water pH in the least tolerant *C. ornata* and highly tolerant *T. pectoralis*. In *C. ornata*, low water Ca^{2+} ($56 \pm 1 \, \mu mol \, L^{-1}$) increased net ion loss relative to high Ca^{2+} ($342 \pm 3 \, \mu mol \, L^{-1}$) water while no effect was observed in *T. pectoralis*. Finally, we find that *T. pectoralis* is among the most acid-tolerant fish species examined to date.

1. Introduction

Environmental acidity poses a significant challenge for most freshwater fishes, causing acid-base balance disturbances and a severe impairment of ion homeostasis at low water pH (Packer and Dunson, 1970; McDonald and Wood, 1981; McDonald et al., 1980; Ultsch et al., 1981). The net loss of ions, primarily Na^+ and Cl^-, results from reduced ion uptake and increased diffusive ion loss at the gills. Reduced ion uptake may result from inhibition of specific transporters, such as vacuolar H^+-ATPase (Gonzalez et al., 2005; Lin and Randall, 1991), or indirectly by the thermodynamically unfavorable condition for Na^+/H^+ exchange at low pH (Gonzalez et al., 2005; Wood, 1989). The Cl^- loss in low pH may be an indirect consequence of lower Na^+ uptake, which can lead to increased H^+ in the plasma that would titrate HCO_3^- to CO_2 which would rapidly re-equilibrate across the gills. This

resulting reduced plasma $[HCO_3^-]$ would reduce Cl^- uptake via Cl^-/HCO_3^- exchangers (Wood, 1989). Increased ion efflux is thought to be caused by displacement of Ca^{2+} by H^+ on the gill membrane tight junctions, where Ca^{2+} normally reduces gill permeability to ions via the paracellular pathway (Hunn, 1985; Madara, 1988). Thus, low ambient Ca^{2+} levels may exacerbate branchial ion loss at low pH. Ultimately, the disturbance of ion balance results in fluid shifts from the extracellular to the intracellular space. The associated reduction in blood plasma volume increases blood viscosity, which combined with stress induced vasoconstriction increases blood pressure and leads to cardiovascular failure (Milligan and Wood, 1982).

Despite the challenges imposed by low pH, regions with the highest freshwater fish diversities in the world, such as the Amazon basin (> 3000 species) (Reis et al., 2016; Val and de Almeida-Val, 1995) and the Mekong delta (800–1200 species) (Baran et al., 2012; Poulsen et al.,

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2004), can be very acidic (Kijne, 2006; Phong et al., 2014; Val and de Almeida-Val, 1995). Tributaries and streams in the Amazon often have pH as low as 3.5 due to the release of organic acids and the water's low buffering capacity (Goulding, 1980; Val & Almeida-Val, 1995). Surface water pH of the Mekong delta can be as low as 3.2 during the rainy season due to leaching of acids from acid sulfate soils that surround 40% of the Mekong delta (Phong et al., 2014). How can so many of these freshwater fishes thrive in these acidic environments? Many of the Amazonian fish species tolerate very low pH by virtue of high resistance to branchial ion loss, and merely experience mild disruptions of Na^+ and Cl^- balance within the first hour of exposure to pH as low as 3 (Gonzalez et al., 1997, 1998, 2005, 2010). Surprisingly, in five Amazonian species tested: aracu (*Leporinus fasciatus*), piranha preta (*Serrasalmus rhombeus*), piranha branca (*Serrasalmus* cf. *holandi*), and pacu (*Myleus* sp.), net ion loss did not change with ambient Ca^{2+} (Gonzalez et al., 1998) as observed in many other species (Duarte et al., 2013; Gonzalez and Wilson, 2001; Gonzalez and Dunson, 1989; Gonzalez et al., 1998; Wilson et al., 1999;). This may suggest that branchial permeability is mediated by other environmental factors, such as dissolved organic carbon (Duarte et al., 2016) in Amazonian species, or that their gill epithelium tight junctions have extremely high binding affinity for Ca^{2+}, preventing H^+ promoted leaching.

While the effect of low pH on ionoregulation of fishes from several other regions have been studied, such studies are lacking for species inhabiting the acidic waters of the Mekong delta. Here, we examine five common Mekong species: *Chitala ornata* (clown knifefish), *Pangasianodon hypophthalmus* (striped catfish), *Osphronemus goramy* (giant gourami), *Trichogaster pectoralis* (snakeskin gourami), and *Monopterus albus* (swamp eel) to determine their tolerance and ionoregulatory ability in low pH. We examined time to loss of equilibrium (LOE) during sustained (4 day) exposure to low pH (pH 3.5) and hypothesized that species with shorter times to LOE would exhibit higher ion losses during acute low pH exposures compared to those with longer times to LOE. Furthermore, we hypothesized that lower ambient Ca^{2+} concentration would increase ion losses at low pH in Mekong species unlike in several of the Amazonian species tested, due to the generally higher Ca^{2+} concentrations in the Mekong (375–632.4 μmol L^{-1}) compared to the Amazon (129–477 μmol L^{-1}) (Li and Bush, 2015).

2. Materials and methods

C. ornata (35–208 g; M = 61 ± 18 g), *P. hypophthalmus* (29–60 g; M = 51 ± 7 g), *O. goramy* (9–24 g, M = 17 ± 2 g), *T. pectoralis* (75–97 g, M = 82 ± 2 g), and *M. albus* (103–131 g, M = 114 ± 4 g) were acquired from commercial fish farms in the Mekong Delta in southern Vietnam. Fish were kept in aerated 300 L tanks at 27 °C in water of pH 8 and ionic concentrations of 236 ± 12 μmol L^{-1} Na^+, 164 ± 2 μmol L^{-1} Cl^-, 34 ± 1 μmol L^{-1} K^+, and 342 ± 3 μmol L^{-1} Ca^{2+} at the College of Aquaculture and Fisheries, University of Can Tho for 3–6 weeks before experiments. All experiments were performed in accordance with national guidelines for the protection of animal welfare in Vietnam.

2.1. Experimental design

2.1.1. Experiment 1: Tolerance to low pH in 5 Mekong fish species

To investigate the relative tolerance of the five species to low pH, *C. ornata* (n = 8), *P. hypophthalmus* (n = 6), *O. goramy* (n = 6), *T. pectoralis* (n = 6), and *M. albus* (n = 4) were placed in an aerated 300 L tank at 27 °C in water pH 8 with ionic composition identical to holding tanks. Then, 0.5 M H_2SO_4 was slowly added to lower water pH to 3.5 over 30 min. The addition of H_2SO_4 would initially result in an increase in pCO2 (not measured) as it titrates HCO_3^-. Due to this, fish were likely exposed to an initial increase in pCO2 that subsided over time. Water was continuousl aerated over the 4 day exposure during which time the fish were regularly monitored for LOE as indicated by an inability to

maintain an upright position in the water column. The time to LOE was noted and fish were euthanized via MS-222 overdose (0.5 g L^{-1} buffered to pH 7 with $NaHCO_3$). Water pH was monitored daily using Radiometer Analytical SAS pH electrode (GK2401C; Cedex, France) connected to a Radiometer PHM 84 (Copenhagen, Denmark) thermostatted to 27 °C and the tank water was maintained at pH = 3.48 ± 0.02 over the 4-day period with subsequent addition of H_2SO_4 as required.

2.1.2. Experiment 2: Effect of water pH and Ca^{2+} on net ion flux in 5 Mekong fish species

Net Na^+, K^+, and Cl^- flux were measured for *C. ornata* (n = 9), *P. hypophthalmus* (n = 9), *O. goramy* (n = 7), *T. pectoralis* (n = 9), and *M. albus* (n = 6) in ambient holding tank water (henceforth referred to as high Ca^{2+} water: 342 ± 3 μmol L^{-1}, pH 8) for 2 h, and then subsequently to high Ca^{2+} water acidified to pH 3.5 and again upon a further reduction in water pH to 3 with further addition of H_2SO_4. These studies identified *C. ornata* and *T. pectoralis* as the most and least sensitive to low pH, respectively. In a separate trial, net Na^+, K^+, and Cl^- fluxes were measured in *C. ornata* (n = 8) and *T. pectoralis* (n = 7) in low Ca^{2+} water (56 ± 1 μmol L^{-1}). Ca^{2+} concentration was reduced by a 6-fold dilution of holding tank water with distilled water followed by addition of sodium chloride to maintain Na^+ and Cl^- concentrations similar to the exposures at high Ca^{2+}.

To conduct the net flux experiments, individuals of each species were placed in aerated rectangular plastic containers (1.5 or 2.5 L depending on the size of the fish) connected to an 80 L recirculating sump system filled with either high or low Ca^{2+} water (pH 8) at 26.8 ± 0.3 °C. Flow rate into containers was approximately 1 L min^{-1} when the system was recirculating. Fish were left to recover overnight before experimentation. To begin net flux measurements, flow to containers was turned off, isolating each chamber from the recirculating system, and each container was aerated to ensure adequate oxygenation for 2 h. A 20 mL water sample was taken from each container at time 0, 1, and 2 h after cessation of water flow for the pH 8 control condition. While containers housing the fish were isolated from the recirculating system for this 2 h duration, water pH in the sump tank was reduced to 3.5 by adding 0.5 M H_2SO_4. The addition of H_2SO_4 would initially elevate water pCO2 and consequently water in the sump was vigorously aerated and stirred with a rod for 2 h prior to fish exposure to aid equilibration of CO_2 with the atmosphere. During the first hour, a small amount of additional H_2SO_4 had to be added to maintain water pH at 3.5, presumably as water pCO2 was reduced, however, no further changes in pH were observed after that. This was verified in a control run where 1 h after the addition of acid, pH remained stable between 3.35 and 3.43 for the next 4 h suggesting that pCO2 had largely equilibrated prior to exposures. We did not measure water pCO2 and we cannot be sure that it was not elevated to some degree which could have unintended consequences on the fish. After the above mentioned 2 h sampling, flow to containers from the sump tank was restored and water pH in each chamber was reduced to 3.5 within 5 mins. After 30 min, flow to the containers was turned off for 2 h and 20 mL water samples were taken at 0, 1, and 2 h of exposure. Again, water pH in the sump was further reduced to 3 with the further addition of H_2SO_4 and vigorous aeration and mixing for 2 h as described above. Following the 2 h sampling period above, flow was restored and water pH in each chamber was further reduced to 3 within 5 min. After 30 min, flow was turned off and 20 mL water samples were taken again at 0, 1, and 2 h. Following the final water sample, fish were transferred to 300 L holding tanks with pH 8 high Ca^{2+} water to recover.

Water Na^+, K^+ and Ca^{2+} concentrations were measured using a flame absorption spectrophotometer. Na^+ and K^+ were measured at Can Tho University using a Sherwood Model 420 (Sherwood Scientific Ltd., Cambridge, UK) and Ca^{2+} was measured in the Department of Zoology at the University of British Columbia using a Spectra AA-240FS (Varian, Australia). Cl^- concentrations were measured using a

colorimetric assay (Zall et al., 1952). Net ion fluxes were calculated as:

$$J_{Net}^{Ion} = \frac{V\left([Ion_1] - [Ion_2]\right)}{Mt}$$

where J_{Net}^{Ion} is net ion flux, V is container volume in liters, $[Ion_1]$ and $[Ion_2]$ are concentrations of an ion measured at time 1 and 2 respectively. M is wet mass of fish in grams, and t is the duration of exposure in hours. In this way, a positive net flux indicates a net uptake of ions and a negative net flux indicates a net loss of ions. All net ion fluxes were calculated as the average of the 2 sequential 1 h mass-normalized fluxes for each water pH.

2.2. Statistical analyses

All statistical analyses were performed in R 3.5.0 (R Core Team, 2013). Residuals of flux data had a leptokurtic distribution and were transformed to a normal distribution using bijective inverse transformation (Goerg, 2016). A linear mixed effects model was then fitted to the data using maximum likelihood method for each ion and species (Bates et al., 2014). The model specified net ion flux as the response variable, pH as the fixed effect variable, mass as a covariate, and subjects nested within pH as the random effect. For *C. ornata* and *T. pectoralis*, pH, Ca^{2+} (high or low), and their interaction terms were assigned as the fixed effect variables. A one-way ANOVA for *P. hypophthalmus*, *O. goramy*, and *M. albus*, and two-way ANOVA for *C. ornata* and *T. pectoralis*, and Tukey's multiple comparison post-hoc test were performed on the fitted linear mixed effects models (Lenth, 2016). Statistical significance was accepted at $p < .05$. All values are expressed as mean $\pm$ SEM.

3. Results and discussion

Our sustained low pH exposures identified *C. ornata* as the least tolerant to low pH, exhibiting the shortest time to LOE in pH 3.5 (100% within 30.5 h). *P. hypophthalmus* and *M. albus* were moderately tolerant, experiencing 50% LOE by the end of the 4-day trial. *O. goramy* and *T. pectoralis* were highly tolerant, with none of the individuals experiencing LOE over the 4-day exposure (Table 1). Based on these results, we expected *C. ornata* to have the highest net ion loss in low pH, followed by *P. hypophthalmus* and *M. albus*, and lowest net ion losses in *O. goramy* and *T. pectoralis*.

Overall, our net ion flux data correlates well with the low pH tolerance we inferred for each species from the time to LOE at pH 3.5. Ion flux varied with pH for *C. ornata* (Fig. 1; Na^+: $F_{2,4} = 14.74$, $p < .01$; K^+: $F_{2,45} = 6.5$, $p < .01$; Cl^-: $F_{2,48} = 5.97$, $p < .01$) and *P. hypophthalmus* (Na^+: $F_{2,26} = 12.26$, $p < .01$; K^+: $F_{2,26} = 10.44$, $p < .01$; Cl^-: $F_{2,25} = 2.26$, $p = .13$), but not for *T. pectoralis* (Na^+: $F_{2,45} = 1.62$, $p = .21$; K^+: $F_{2,45} = 2.70$, $p = .08$; Cl^-: $F_{2,45} = 3.11$, $p = .06$), *O. goramy* (Na^+: $F_{2,21} = 3.23$, $p = .06$; K^+: $F_{2,21} = 0.22$, $p = .81$; Cl^-: $F_{2,21} = 0.07$, $p = .93$), and *M. albus* (Na^+: $F_{1,12} = 0.45$, $p = .52$; K^+: $F_{1,12} = 2.66$, $p = .13$; Cl^-: $F_{1,12} = 0.01$, $p = .93$). Mass had a significant inverse effect on Na^+ flux for *C. ornata* and Cl^- flux

for *O. goramy*. For *C. ornata*, this result is driven by a single individual (m = 208 g) that was much larger than the rest of the individuals (m = 35-60 g). Likewise, for *O. goramy*, the relationship is driven by the two smallest individuals used in our study (9 and 10 g). Thus, while mass had a significant effect on ion flux in *C. ornata* and *O. goramy*, this did not alter our conclusion that *C. ornata* is the least tolerant and that *O. goramy* is one ofthe most tolerant species that we examined. In control water of pH 8, K^+ flux was close to zero for all species. Apart from *M. albus*, all species displayed a net loss of both Na^+ and Cl^-, possibly due to stress associated with the holding conditions. There were no significant differences in ion flux for any species between pH 8 and 3.5 (Na^+, K^+, Cl^-; *C. ornata*: $p = .34, 0.85, 0.87$; *P. hypophthalmus*: $p = .77, 0.97, 0.87$; *M. albus*: $p = .52, 0.1, 0.99$; *O. goramy*: $p = .09, 0.9, 0.94$; *T. pectoralis*: $p = .26, 0.73, 0.98$). However, *C. ornata* experienced a mean increase in Na^+ loss when water pH was reduced to 3.5, which was not observed in the moderately sensitive *P. hypophthalmus* and *M. albus* (Fig. 1A, B, E). Furthermore, assuming a continuous net efflux rate of those measured at a water pH 3.5, *C. ornata* ($-1235 \, nmol \, g^{-1} \, h^{-1}$) and *P. hypophthalmus* ($-452 \, nmol \, g^{-1} \, h^{-1}$) would lose most of their extracellular fluid Na^+ in 20 and 46 h, respectively (Fig. 1), assuming that extracellular fluid makes up 18% of body mass (Olson, 1992) and extracellular Na^+ concentration is 135 mM like in other teleosts (Booth et al., 1988; Gam et al., 2017, 2018; Gonzalez and Dunson, 1987). Presumably, this rate of extracellular Na^+ loss could be correlated with time to LOE at the same water pH. In *C. ornata*, LOE is reached much before 20 h, while *P. hypophthalmus* experienced LOE much later than 46 h. In the former this is not surprising as LOE would be experienced long before all Na^+ was lost from plasma and we cannot rule out additional effects of elevated pCO_2 at this time point. However, that *P. hypophthalmus* only exhibit 50% LOE at 96 h implies that the net Na^+ loss rate measured during acute exposure is not representative of that during longer exposure. Regardless, *P. hypophthalmus* is more tolerant than *C. ornata* to the combined low pH and elevated pCO_2 conditions.

Net Na^+ losses were greatly increased when pH was reduced to 3 for *C. ornata* ($p < .01$) and *P. hypophthalmus* ($p < .01$), whereby Na^+ loss was 4 and 6 times greater than in pH 8, respectively (Fig. 1A, B). *P. hypophthalmus* also experienced an over 90-fold increase in K^+ loss at pH 3 relative to 8 ($p < .01$). While not statistically significant, *C. ornata* also experienced a nearly 8-fold increase in K^+ loss ($p = .06$). High K^+ loss is a particularly relevant indicator of osmotic disruption (McDonald et al., 1980; Milligan and Wood, 1982; Audet and Wood, 1988). Thus, the pronounced K^+ loss at pH 3 in both species may indicate a severe disruption to ionoregulatory homeostasis. *P. hypophthalmus* experienced a much more substantial K^+ loss compared to *C. ornata*, suggesting that *P. hypophthalmus* may be more sensitive to low pH than net efflux of Na^+ and Cl^- would imply.

Unlike the other species, *M. albus* showed visible signs of severe distress as indicated by continuous, forceful writhing within the container within 30 min at pH 3 that was atypical of the sedentary behavior observed at higher pH values, and we consequently terminated the pH 3 exposure in this species. This pronounced avoidance behavior at pH 3 and a time to 50% LOE of 54 h during sustained exposure to pH 3.5, despite negligible net ion loss during acute exposure to pH 3.5, indicates that *M. albus* has a lower tolerance to acidic water than suggested by net ion loss. This may be associated with its obligate air-breathing life history and associated reduced gill surface area (Liem, 1947). However, our evidence for low tolerance at pH 3 is based on behavioral rather than physiological evidence and it remains to be seen if net ion flux increases at pH 3.

The tolerant *O. goramy* and *T. pectoralis* exhibited low ion losses at all water pH values, consistent with other tolerant species (Duarte et al., 2013; Gonzalez and Dunson, 1987; Gonzalez and Wilson, 2001; Gonzalez et al., 1998). Taken together with our sustained low pH exposure, it would then seem that both species would have long term tolerance to pH 3 waters, similar to other species previously examined (Gonzalez et al., 1998; Gonzalez and Wilson, 2001; Gonzalez and
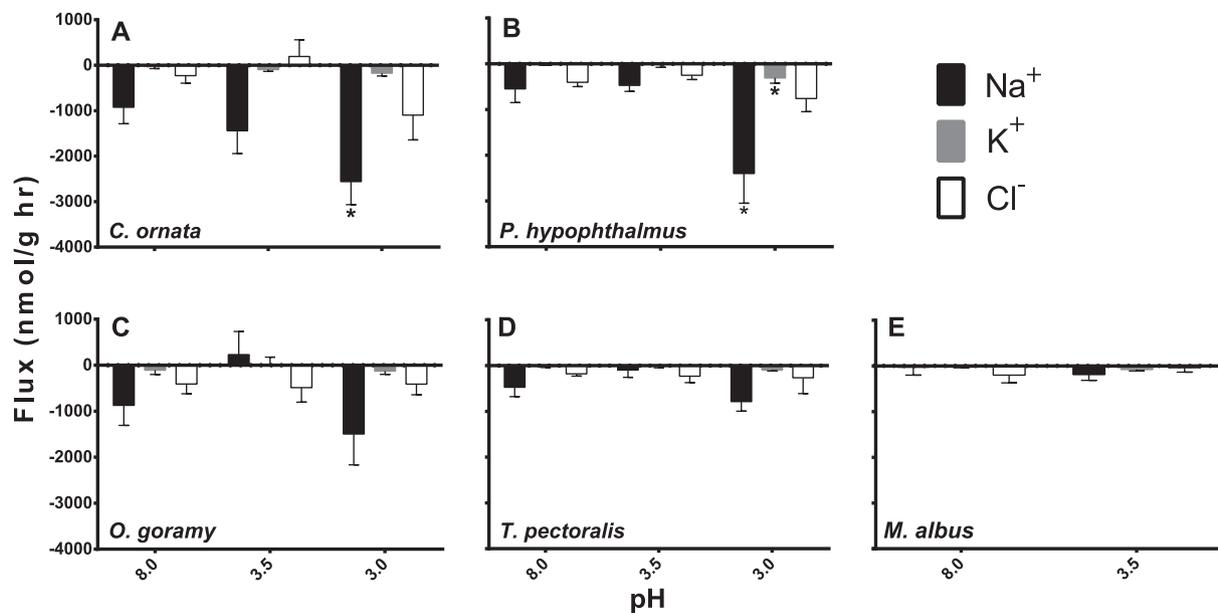
Table 1
Percentage of *C. ornata* (n = 8), *P. hypophthalmus* (n = 6), *O. goramy* (n = 6), *T. pectoralis* (n = 6), and *M. albus* (n = 4) maintaining equilibrium in water pH 3.5 over time.

Time (hours)	Maintaining equilibrium (%)				
	C. ornata	*P. hypophthamus*	*O. goramy*	*T. pectoralis*	*M. albus*
0	100	100	100	100	100
8.5	37.5	100	100	100	100
30.5	0	83.3	100	100	75
54.5	0	83.3	100	100	50
94	0	50	100	100	50

Fig. 1. Effects of water pH on net flux of Na^+, K^+, and Cl^- ions for *C. ornata* (A), *P. hypophthalmus* (B), *O. goramy* (C), *T. pectoralis* (D), and *M. albus* (E). *M. albus* displayed visible distress at pH 3 treatment and were thus only exposed to pH 8 and 3.5. * indicates significant difference from fluxes at pH 8.
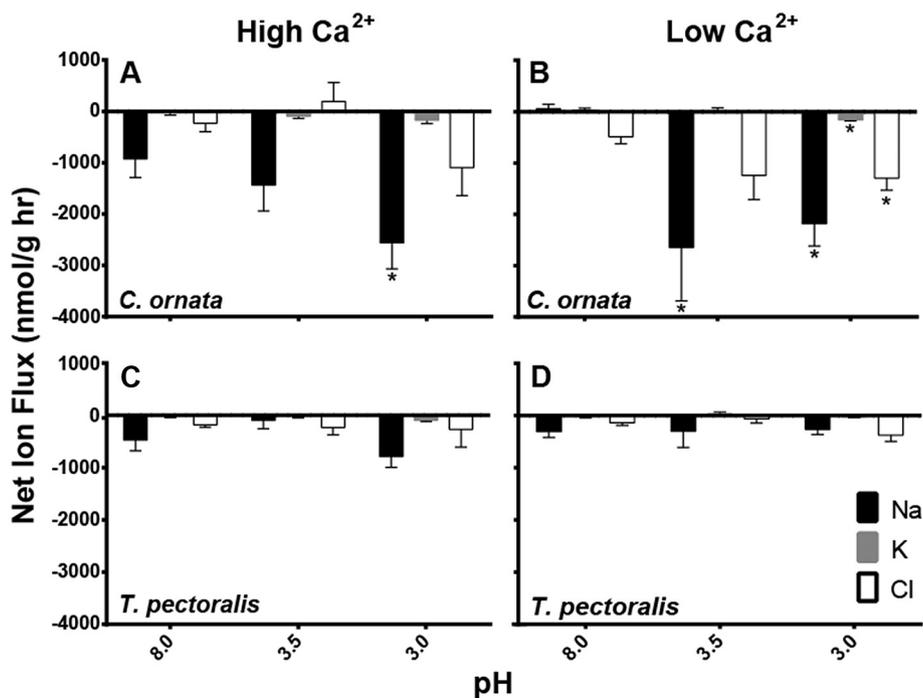

Fig. 2. Effect of water pH on net ion flux in high and low Ca^{2+} of *C. ornata* (A, B) and *T. pectoralis* (C, D) in high (A, C) and low Ca^{2+} (B, D) water. A and C are replotted from Fig. 1. * indicates significant difference from fluxes at pH 8.

Dunson, 1987). However, a long term (≥ 7 day) pH 3 exposure is necessary to determine this. Furthermore, *T. pectoralis* in low Ca^{2+} at pH 3 and 3.5 water exhibited lower Na^+ losses (Fig. 2) compared to the Amazonian angelfish (*Pterophyllum scalare*) and cardinal tetra in similar $[Ca^{2+}]$ (50 µmol L^{-1}) and pH 3.4 (Gonzalez and Wilson, 2001). *T. pectoralis* also had lower Na^+, Cl^-, and K^+ losses in our low Ca^{2+}, pH 3 water compared to the Amazonian fish species: aracu, piranha preta, piranha branca, and pacu in 100 µmol L^{-1} Ca^{2+}, pH 3 water (Gonzalez et al., 1998). Given the latter study had 2-fold higher water $[Ca^{2+}]$, this implies that *T. pectoralis* is more tolerant to low pH than these Amazonian species and is among the most low pH tolerant species currently known. Only discus (*Symphysodon discus*) has lower Na^+ loss than *T.*

pectoralis at pH 3.5 (Duarte et al., 2013), and it remains to be investigated whether discus is also more tolerant than *T. pectoralis at* pH of 3. However, our study used water with Na^+, K^+, and Cl^- compositions higher than that used for the Amazonian fish studies, which would result in a lower gradient for diffusive ion losses. To truly compare the low pH tolerance of Mekong and Amazonian fishes, future studies must use water with the same ionic compositions.

Low Ca^{2+} water exacerbated the effects of low pH on ion loss in *C. ornata* (the least tolerant species), but not in *T. pectoralis* (the most tolerant species; Fig. 2). However, only Cl^- flux in *C. ornata* differed between low and high Ca^{2+} groups (*C. ornata*: Na^+: $F_{1,45} = 0.16$, $p = .69$, K^+: $F_{1,45} = 0.96$, $p = .33$, Cl^-: $F_{1,48} = 11.37$, $p < .01$; *T.*

pectoralis: Na$^+$: F$_{1,45}$ = 0.51, p = .48, K$^+$: F$_{1,45}$ = 3.18, p = .08, Cl$^-$: F$_{1,16}$ = 0.60, p = .45). In addition to the significantly higher loss of Na$^+$ at low Ca^{2+}, pH 3 (p < .01), *C. ornata* exposed to low Ca^{2+}, pH 3.5 experienced an increased Na$^+$ loss (p < .01) that it did not experience in high Ca^{2+}, pH 3.5. Similarly, *C. ornata* experienced increased Cl$^-$ loss in low Ca^{2+}, pH 3.5 (p = .04) that it did not experience in high Ca^{2+}, pH 3.5. Additionally, the magnitude of Na$^+$ and Cl$^-$ losses in low Ca^{2+}, pH 3.5, were similar to losses measured at both low and high Ca^{2+}, pH 3. This suggests that our measured ion losses were maximal and that ambient Ca^{2+} served to shift the threshold pH at which *C. ornata* experienced maximal ion losses. It would be interesting to measure ion fluxes of *C. ornata* in low pH with higher ambient Ca^{2+} than tested in our study. Similar to high Ca^{2+}, there were no differences in ion losses in *T. pectoralis* in low Ca^{2+}, pH 3.5 (Na$^+$: p = .98, K$^+$: p = .69, Cl$^-$: p = .83) or 3 (Na$^+$: p = .99, K$^+$: p = .98, Cl$^-$: p = .83). This may suggest that branchial permeability is not mediated by Ca^{2+} in *T. pectoralis* like in some Amazonian species: aracu, piranha preta, piranha branca, and pacu (Gonzalez et al., 1998), or that our low Ca^{2+} treatment of 55.6 µmol L^{-1} was still high enough to prevent ion loss (Freda and McDonald, 1988). Regardless, our results add to the evidence that *T. pectoralis* is highly adapted to tolerate low pH.

The tolerance to acidic water of each species is consistent with their natural habitat. The leaching of sulfates from acid sulfate soils surrounding the Mekong is greatest, and consequently surface water pH the lowest, in flood plains at the start of the rainy season (Minh et al., 1997). While *C. ornata* and *P. hypophthalmus* typically inhabit the main Mekong river and avoid flood plains during the dry season and the beginning of the rainy season (Poulsen et al., 2004), *O. goramy* and *T. pectoralis* only leave the flood plains when the water recedes (Valbo-Jørgensen et al., 2009). *C. ornata* and *P. hypophthalmus* only migrate to the flood plains during the flooding season when pH has increased above 5 due to dilution by rainwater (Minh et al., 1997). It appears then, that *C. ornata* and *P. hypophthalmus* avoid, while *O. goramy* and *T. pectoralis* withstand low pH in their natural habitat. It is noteworthy that *O. goramy* and *T. pectoralis* (Anabantiformes – Osphronemidae) are the most closely related species examined in our study; all other species we examined: *C. ornata* (Osteoglossiformes – Notopteridae), *P. hypophthalmus* (Siluriformes – Pangasiidae), and *M. albus* (Synbranchiformes – Synbranchidae) are from different families and orders. Prior studies on Characiformes and Cichliformes species identify that these two orders have highly conserved but different ionoregulatory strategies that allow them to tolerate low pH environments, suggesting that an ancestral physiological trait allowed species within these orders to subsequently inhabit acidic freshwater habitats (Gonzalez et al., 2017, 2018). Our present study and the evidence from other groups of fishes implies that Osphronemid fishes may also have had an ancestral physiological trait that allowed them to tolerate low pH habitats. However, due to the limited number of species that we examined, it remains difficult to establish whether behavioral ecology and subsequent evolution of low pH tolerance or ancestral physiology lead to niche division among the Mekong fishes. This could be addressed in part with further studies on low pH tolerance of other closely related species that live in different pH environments in conjunction with lab reared F1 and F2 populations; clearly an area ripe for further study.

Competing interests

No competing interests declared.

Funding

Summary statement

We assessed low pH tolerance in five fish species of the Mekong, by measuring the ability to regulate ion balance in low water pH at low and high ambient calcium.

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