



Characterization of Na⁺ transport to gain insight into the mechanism of acid-base and ion regulation in white sturgeon (*Acipenser transmontanus*)



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ABSTRACT

Freshwater fish actively take up ions via specific transporters to counter diffusive losses to their hypotonic environment. While much is known about the specific mechanisms employed by teleosts, almost nothing is known about the basal fishes, such as white sturgeon (*Acipenser transmontanus*) which may offer insight into the evolution of osmo- and ionoregulation in fishes. We investigated Na⁺ uptake in juvenile white sturgeon in the presence and absence of transporter inhibitors. We found that sturgeon acclimated to 100 μmol l⁻¹ Na⁺ have Na⁺ uptake kinetics typical of teleosts and that a Na⁺/H⁺ exchanger (NHE) is the predominant transporter for Na⁺ uptake. White sturgeon are tolerant to hypercarbia-induced respiratory acidoses and recover blood pH (pH_e) at 1.5 kPa PCO₂ but not at higher PCO₂ (6 kPa PCO₂) where they preferentially regulate intracellular pH (pH_i). It was hypothesized that during exposure to hypercarbia Na⁺ uptake would increase at CO₂ tensions at which fish were capable of pH_e regulation but decrease at higher tensions when they were preferentially regulating pH_i. We found that Na⁺ uptake did not increase at 1.5 kPa PCO₂, but at 6 kPa PCO₂ Na⁺ uptake was reduced by 95% while low water pH equivalent to 6 kPa PCO₂ reduced Na⁺ uptake by 71%. Lastly, we measured net acid flux during hypercarbia, which indicates that net acid flux is not associated with Na⁺ uptake. These findings indicate Na⁺ uptake in sturgeon is not different from freshwater teleosts but is sensitive to hypercarbia and is not associated with pH_e compensation during hypercarbia.

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1. Introduction

Ion homeostasis in freshwater fishes is achieved by using specialized branchial transport mechanisms for active ion uptake (primarily Na⁺ and Cl⁻) from an environment that is hypotonic to the blood, and a tight gill epithelium to minimize ionic diffusive losses (Evans, 2011; Hwang et al., 2011). At the gills of freshwater fishes, apical Na⁺ uptake is coupled to acid excretion and can involve different apical transport proteins, depending on the species and/or the environment they inhabit. The latter may consist of a Na⁺ channel or acid-sensing ion channel (ASIC) electrically linked to H⁺ extrusion by a V-ATPase (Dymowska et al., 2014), an electro-neutral Na⁺/H⁺ (or Na⁺/NH₄⁺ or H⁺ + NH₃) exchanger (NHE) (Hwang et al., 2011), Cl⁻-dependent Na⁺ uptake via a Na⁺-Cl⁻ co-transporter (NCC) (Hwang et al., 2011) or Na⁺-K⁺-2Cl⁻

co-transporter (NKCC) (Brix and Grosell, 2012; Hiroi et al., 2005), or some combination of these mechanisms. Common to these mechanisms for Na⁺ uptake is the Na⁺/K⁺-ATPase (NKA) in the basolateral membrane of gill ionocytes which exports Na⁺ into the blood and contributes to the electrochemical gradient that drives Na⁺ uptake across the apical membrane (Hwang et al., 2011). In dilute freshwater, a Na⁺ channel or ASIC associated with H⁺-ATPase is believed to be the most thermodynamically favorable (Parks et al., 2008), however a number of studies have demonstrated that Na⁺ uptake occurs in conjunction with an NHE in some freshwater fishes [e.g. zebrafish *Danio rerio* (Kumai and Perry, 2011; Yan et al., 2007); medaka *Oryzias latipes* (Wu et al., 2010); Osorezan dace *Tribolodon hakonensis* (Hirata et al., 2003); and pupfish *Cyprinodon variegatus hubbsi* (Brix et al., 2015; Brix and Grosell, 2012)]. Recently, it has been shown that NHEs may operate in low Na⁺ environments (i.e. freshwater) when they are associated with Rhesus (Rh) proteins, transporting ammonia (Kumai and Perry, 2011; Wu et al., 2010; Yan et al., 2007) or with a basolateral membrane sodium bicarbonate co-transporter (NBC). In the latter, carbonic anhydrase (CA) generates H⁺ and HCO₃⁻ from metabolically produced CO₂, and HCO₃⁻ is exported by the NBC across the basolateral membrane leaving intracellular H⁺ to drive the apical NHE (Hirata et al., 2003; Scott et al., 2005).

Abbreviations: ASIC, acid-sensing sodium channel; CA, carbonic anhydrase; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; kPa, unit of pressure; MRC, mitochondrion-rich cell; NBC, Na⁺, HCO₃⁻ co-transporter; NHE, Na⁺/H⁺ exchanger; Rh, Rhesus protein; PCO₂, partial pressure of CO₂; pH_e, extracellular pH; pH_i, intracellular tissue pH; PO₂, partial pressure of O₂.

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Most of the studies characterizing the mechanisms of Na^+ uptake in freshwater fishes have been conducted on teleosts, a highly diverse group, comprising the vast majority of extant fish species. Surprisingly, however, nothing is known about the basal fishes, which may offer insight into the evolution of osmo- and ionoregulation in fishes (and vertebrates). We were interested in examining Na^+ uptake in one of these basal fishes, white sturgeon *Acipenser transmontanus* for this reason. Sturgeon belong to the order Acipenseriformes, which are believed to have diverged from the Neopterygii (which includes teleosts) approximately 343–372 million years ago (Betancur-R et al., 2013; Hedges and Kumar, 2009). White sturgeon inhabit river systems along the Pacific coast of North America containing dilute freshwater, where Na^+ concentrations in the Fraser (Swain et al., 1998) and Columbia (City of Trail, 2013; City of Revelstoke, 2010) Rivers, for example, may be $<80 \mu\text{mol l}^{-1}$. Similar to salmonids, sturgeon spawn in freshwater and juveniles are believed to remain in freshwater for several months or years (McEnroe and Cech, 1985), but depending on the species may spend some of their life in seawater; salinity tolerance appears to be associated with fish size, but there are likely to be considerable differences between populations (Allen et al., 2014; Mojazi Amiri et al., 2009).

Sturgeon are relatively inactive and have low metabolic rates relative to other fishes (Baker and Brauner, 2012; Fitzgibbon et al., 2008). Low metabolic rate is generally associated with low Na^+ loss and therefore, low Na^+ uptake rate in fishes (Gonzalez and McDonald, 1994). Thus, we hypothesized that juvenile sturgeon would have a low Na^+ uptake rate under routine conditions that would be accomplished by a low Na^+ capacity, but high affinity system. We also hypothesized that Na^+ uptake would be driven by a Na^+ channel or ASIC/ H^+ -ATPase based on observations by Baker et al. (2009) showing weak apical NHE3 staining and the presence of apical V-ATPase at the gills, and because a Na^+ channel or ASIC/ H^+ -ATPase system would be more thermodynamically favorable (Parks et al., 2008) in the relatively low Na^+ water that sturgeon inhabit. These hypotheses were tested in Series 1 using experiments designed to investigate Na^+ uptake transport mechanisms. Series 1 experiments examined Na^+ uptake kinetics and the effect of specific transporter inhibitors [phenamil (Na^+ channel inhibitor), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; NHE inhibitor); bumetanide (NKCC inhibitor); ethoxzolamide (CA inhibitor); 4',6-diamidino-2-phenylindole (DAPI; ASIC inhibitor)] in white sturgeon. Small juvenile fish (~2–5 g) were used, as they are likely to be exclusively freshwater under natural conditions and are a suitable size for radioisotope experiments.

Sturgeon are one of the most CO_2 tolerant fishes known and are able to withstand acute exposure to increased water PCO_2 (hypercapnia) of 12 kPa for 48 h (Baker and Brauner, 2012). Interestingly, at low CO_2 tensions (<1.5 kPa) they are able to compensate for reductions in extracellular pH (pH_e), but not at high CO_2 tensions (>3 kPa) (Baker et al., 2009; Shartau et al., 2016). Respiratory acidosis induced by hypercapnia have been demonstrated to increase Na^+ uptake in some fish, likely in association with increased H^+ efflux and pH_e compensation as observed in rainbow trout (*Oncorhynchus mykiss*) (Perry et al., 1987), brown bullhead (*Ictalurus nebulosus*) (Goss et al., 1992) and arctic grayling (*Thymallus arcticus*) (Cameron, 1976). Similarly, hypercapnia exposure leads to increased extracellular $[\text{Na}^+]$ in blue crab (*Callinectes sapidus*), channel catfish (*Ictalurus punctatus*) (Cameron and Iwama, 1987), Japanese flounder (*Paralichthys olivaceus*), yellowtail (*Seriola quinqueradiata*) and star-spotted dogfish (*Mustelus manazo*) (Hayashi et al., 2004) possibly implying an association between Na^+ uptake and H^+ excretion. As there are differences in pH_e regulation during exposure to hypercapnia at low and high CO_2 tensions in sturgeon, we were interested in how Na^+ uptake characteristics would be affected and whether this may contribute to the different strategies of pH_e regulation during different levels of hypercapnia. We hypothesized that Na^+ uptake would increase during hypercapnia exposure at ≤ 1.5 kPa PCO_2 when pH_e compensation occurs, but at higher CO_2 tensions, Na^+ uptake would be reduced due to the effect of low pH hindering H^+ extrusion

(Lin and Randall, 1995). These hypotheses were tested in Series 2 using experiments designed to investigate the effect of acid-base disturbances on Na^+ uptake and net acid flux.

Series 2 was organized into three groups of experiments. The first experiment in Series 2 (Series 2.1) was performed to corroborate that there was a difference in pH_e compensation between low (1.5 kPa PCO_2) and high (6 kPa PCO_2) hypercapnia exposure in juvenile white sturgeon. The second set of experiments (Series 2.2) examined Na^+ uptake during various acid-base disturbances. Here, we measured Na^+ uptake during 3 h hypercapnia exposure to 0.75, 1.5, 3 or 6 kPa PCO_2 , followed by measurements of Na^+ uptake at 1.5 kPa PCO_2 over 12 h to assess if Na^+ uptake changes during pH_e recovery. Next, since hypercapnia lowers water pH, we wanted to distinguish between the effect of low water pH and hypercapnia on Na^+ uptake; therefore, fish were exposed to the equivalent water pH (via H_2SO_4 addition to the water) as the CO_2 exposures for 3 h and Na^+ uptake was measured. Finally, we wanted to assess if the source of CO_2 [external (hypercapnia) or internal (hypercapnia)] affects Na^+ uptake; to address this, fish were exposed to hyperoxia (high environmental O_2) for 3 h, which induces retention of metabolically produced CO_2 and creates an internally sourced respiratory acidosis (hypercapnia) (Wood and LeMoigne, 1991). The third set of experiments in Series 2 (Series 2.3) measured net acid flux during hypercapnia and low water pH to investigate the relationship between Na^+ uptake and net acid flux during these acidosises.

2. Methods

2.1. Animal acquisition and holding

White sturgeon (*A. transmontanus*) were reared at the International Centre for Sturgeon Studies at Vancouver Island University (Nanaimo, British Columbia, Canada) in dechlorinated water [$61 \mu\text{mol l}^{-1} \text{Na}^+$, $69 \mu\text{mol l}^{-1} \text{Cl}^-$ (City of Nanaimo, 2015), pH ~6.6–6.8 (Mojazi Amiri et al., 2009)] and transported to the Department of Zoology aquatic facilities at the University of British Columbia (Vancouver, British Columbia, Canada) at the juvenile stage (~3 months old, 1–4 g). Fish were held in flow-through dechlorinated City of Vancouver tap water [~ 55 – $84 \mu\text{mol l}^{-1} \text{Na}^+$, $73 \mu\text{mol l}^{-1} \text{Cl}^-$, $7 \mu\text{mol l}^{-1} \text{Mg}^{2+}$, $89 \mu\text{mol l}^{-1} \text{Ca}^{2+}$ (Metro Vancouver, 2015), pH 6.3, $3 \mu\text{mol l}^{-1} \text{HCO}_3^-$] for 5–6 weeks before experiments and fed *ad libitum* every second day and starved for 48 h before experiments unless otherwise indicated. Animal transport was conducted in accordance with federal and provincial regulations (BC ITC Transfer no: 13531). All experiments were approved by the University of British Columbia animal care committee (animal care no: A11-0235).

2.2. Experimental protocols

2.2.1. Series 1: characterization of Na^+ uptake

2.2.1.1. Na^+ uptake kinetics. The Na^+ uptake kinetics of juvenile white sturgeon (1.36–3.52 g) were determined in fish at 15 °C. Uptake rates were measured at eight water Na^+ concentrations ranging from 10 to $890 \mu\text{mol l}^{-1} \text{Na}^+$. At each Na^+ concentration five fish were placed in 1000 ml of a defined medium ($480 \mu\text{mol l}^{-1} \text{CaSO}_4$, $150 \mu\text{mol l}^{-1} \text{MgSO}_4$, $100 \mu\text{mol l}^{-1} \text{KHCO}_3$, pH 7.0) to which the desired concentration of NaCl was added. Flux water was continuously aerated to ensure sufficient oxygenation. Fish were allowed to acclimate for 10 min and then 1–2 μCi of ^{22}Na (depending on the ambient Na^+ concentration) was added to the solution. The flux solution was sampled after 1 min for measurements of $[\text{Na}^+]$ (2 ml) and ^{22}Na (1 ml). The total flux exposure time ranged from 1.5 to 2.5 h, depending on the ambient Na^+ concentration used. In all cases, the internal specific activity was $<1\%$ of the external specific activity such that correction for backflux was unnecessary (Maetz, 1956). At the end of the exposure time, water samples for $[\text{Na}^+]$ and ^{22}Na activity were collected, fish were removed from the

exposure medium, double rinsed in a 100 mmol l⁻¹ Na⁺ solution to displace any loosely bound ²²Na, then euthanized in a 100 mmol l⁻¹ Na⁺ solution containing MS-222 (0.5 g l⁻¹ MS-222 buffered with 1 g l⁻¹ NaHCO₃) and blotted dry. Fish were then placed in pre-weighed empty scintillation vials which were weighed to the nearest 0.1 mg and then counted individually for radioactivity.

2.2.1.2. Pharmacological inhibitor experiments. Sturgeon acclimated to dechlorinated City of Vancouver tap water were used to measure Na⁺ uptake in the presence and absence of different pharmacological inhibitors. It was hypothesized that juvenile sturgeon use a Na⁺-channel or ASIC/H⁺-ATPase system for Na⁺ uptake across the apical membrane; therefore, the pharmacological experiments were designed to test this hypothesis and identify the most likely transport proteins involved in Na⁺ uptake with the fewest experiments possible to minimize the number of fish used.

The first inhibitor used was phenamil, a potent Na⁺ channel inhibitor that has low affinity for NHEs (Kleyman and Cragoe, 1988). For the phenamil treatment, 8 fish were exposed in 1000 ml of defined media (see above) for 10 min containing 100 μmol l⁻¹ NaCl. Phenamil dissolved in dimethyl sulfoxide (DMSO) was then added at a final concentration of 2 × 10⁻⁵ mol l⁻¹ and 0.1% DMSO. Five minutes following inhibitor/DMSO addition, 1 μCi of ²²Na was added and fish were left for 1 h. At the beginning and end of the exposure period, a 1 ml sample was collected for measurement of [Na⁺] and ²²Na activity. At the end of the exposure period, fish were treated as described in the Na⁺ uptake experiments above. A similar experimental design was used in subsequent inhibitor experiments. Fish were exposed to 4 × 10⁻⁵ mol l⁻¹ EIPA [5-(N-ethyl-N-isopropyl)-amiloride], which is a potent NHE inhibitor with low affinity for Na⁺ channels (Kleyman and Cragoe, 1988). To investigate whether Na⁺ uptake was chloride-dependent, fish were exposed to 10⁻⁴ mol l⁻¹ bumetanide [sodium potassium chloride co-transporter (NKCC) inhibitor] and in a separate experiment to Cl⁻-free media (50 μmol l⁻¹ Na₂SO₄ instead of 100 μmol l⁻¹ NaCl). Next, we investigated the possible role of carbonic anhydrase (CA) in Na⁺ uptake by exposing fish to 10⁻⁴ mol l⁻¹ ethoxzolamide (6-ethoxy-1,3-benzothiazole-2-sulfonamide), a potent CA inhibitor (Brix and Grosell, 2013). These inhibitor treatments were compared to a control carrier treatment with only 0.1% DMSO; there was no effect of DMSO on Na⁺ uptake ($t_{10} = 0.2297$, $P = 0.8229$). Finally, to assess the presence/absence of acid-sensing ion channels (ASIC), fish were exposed to 10⁻⁶ mol l⁻¹ DAPI (4',6-diamidino-2-phenylindole), an ASIC inhibitor (Dymowska et al., 2014); another control was conducted as the DAPI treatment was performed at a later date than the other inhibitor treatments.

To evaluate the potential presence of a NHE-Rh metabolon, we followed the general experimental design of Kumai and Perry (2011), Shih et al. (2012) and Brix et al. (2015) to manipulate independently and in combination Na⁺ and total ammonia (T_{amm}) gradients across the gills and then measured the effects of these manipulations on concurrent Na⁺ influx and T_{amm} excretion. The following five treatments were used to manipulate gradients: (i) Fed – fish were fed to satiation twice per day for 2 d, including a meal 3 h prior to experimentation with the objective of increasing the efflux of T_{amm} from the fish; (ii) pH 4.0 – fish were acclimated to pH 4.5 for 1 week, then placed in exposure water that was adjusted to pH 4.0 (water pH was adjusted using HNO₃); (iii) EIPA – fish were exposed to 5 × 10⁻⁵ mol l⁻¹ EIPA during the Na⁺/T_{amm} flux, but were not acclimated to this condition; (iv) Control – defined medium; (v) Carrier control – as DMSO interferes with the colorimetric assay used to measure T_{amm}, (2-hydroxypropyl)-β-cyclodextrin was used instead to solubilize EIPA (Brix et al., 2015). For each treatment, 8 fish were placed individually in 160 ml of defined media containing 100 μmol l⁻¹ Na⁺. Fish were allowed to acclimate to this media for 10 min after which 1 μCi of ²²Na was added to the solution. The flux solution was sampled after 1 min for initial measurements of [Na⁺] (2 ml), ²²Na activity (1 ml) and T_{amm} (1 ml). The total

flux exposure period ranged from 3.5 to 4.5 h. At the end of the exposure period, the flux solution was sampled for final measurements of [Na⁺], ²²Na activity and T_{amm}, and fish were removed from exposure media as indicated above and counted individually for radioactivity.

2.2.2. Series 2: acid-base disturbances, Na⁺ uptake and net acid flux

2.2.2.1. Effect of hypercarbia on blood pH (pH_e). To verify that changes in blood pH (pH_e) in these smaller fish are the same as those reported by Baker et al. (2009) in larger (>500 g) sturgeon during control and hypercarbic conditions, fish were carefully transferred to a 15 l plastic box that was aerated with either air (0.04 kPa PCO₂; control), 1.5 or 6 kPa PCO₂; PO₂ was maintained at 21 kPa, balanced with N₂. Fish that were exposed to hypercarbia were sampled at either 3 or 24 h, controls were sampled at 3 h. At sampling time, fish were immediately anesthetized (0.4 g l⁻¹ MS-222 buffered 0.8 g l⁻¹ with NaHCO₃ that was aerated with the same gas mix as the treatment) and blood sampled via cardiac puncture (100–300 μl) using a heparinized 1 ml syringe, which was placed on ice until pH_e was measured. Blood pH was measured using a thermostated capillary pH electrode (model BMS 3 MK 2; Radiometer). All desired gas mixtures of CO₂, O₂ and N₂, in this and following experiments, were mixed using a DIGAMIX 275 6KM 422 Woesthoff pump (Bochum, Germany).

2.2.2.2. Na⁺ uptake during acid-base disturbances. To examine the effect of acid-base disturbances on Na⁺ uptake, white sturgeon were subjected to external and internal acidosis. To achieve an external acidosis, water pH was reduced with and without elevated CO₂ (hypercarbia and low water pH, respectively) and an internal acidosis was imposed with and without reduction in environmental pH (hypercarbia and hyperoxia, respectively). For all treatments, 10 fish were placed in 1000 ml of dechlorinated tap water that was aerated for 1 h before treatment; unless otherwise specified, fish were subjected to the desired treatment for 3 h and PO₂ was maintained at 21 kPa. Na⁺ uptake was measured by adding 1 μCi of ²²Na in the final hour of CO₂ exposure. This time point was chosen in order to measure Na⁺ uptake associated with pH compensation following the onset of the acidosis.

To investigate potential differences in Na⁺ uptake at various CO₂ tensions, fish were exposed to air (control), 0.75, 1.5, 3 or 6 kPa PCO₂; to investigate if Na⁺ uptake changes during pH_e recovery, fish were exposed to 1.5 kPa PCO₂ for 6 or 12 h. The effect of reduced water pH on Na⁺ uptake in the absence of CO₂ was measured in fish exposed to a water pH of 5.9, 5.5 or 5.2, which was adjusted through the addition of H₂SO₄. These water pH values correspond with those measured during exposure to 1.5, 3 and 6 kPa PCO₂, respectively. Finally, we wanted to assess if Na⁺ uptake varied when the source of CO₂ was internal (hyperoxia induced hypercapnia) as opposed to external (hypercarbia) as the former does not change water pH while the latter does, which may affect apical branchial ion transporters. Fish were subjected to hyperoxia (80 kPa PO₂ and 20 kPa PN₂), which creates a respiratory acidosis approximately equivalent to 1–1.5 kPa PCO₂ (Wood and LeMoigne, 1991).

2.2.2.3. Net acid flux during hypercarbia and low water pH. To investigate the relationship between Na⁺ influx and acid excretion, we measured net acid flux during hypercarbia and low water pH. Fish were subjected to 1.5 or 6 kPa PCO₂, or water pH of 5.9 and 5.2, which correspond with those measured during exposure to 1.5 and 6 kPa PCO₂, respectively. Net acid excretion was determined by measuring titratable acid flux and total ammonia (T_{amm}) flux, as described below.

2.3. Analytical methods, calculations and statistical analysis

Total Na⁺ in water samples was measured by atomic absorption spectrophotometry (Varian Spectra AA-220, Mulgrave, Victoria, Australia). Radioactivity of water and fish samples were measured for ²²Na

activity using a gamma counter (Wallac 1470 Wizard, PerkinElmer, Finland). Rates of Na^+ uptake as measured by the appearance of radioactivity in the fish were calculated as previously described by Boisen et al. (2003). Water T_{amm} was measured by a micro-modified colorimetric method (Verdouw et al., 1978). Water pH was measured by a glass electrode (Radiometer Analytical SAS pH electrode; GK2401C, Cedex, France or Orion 8302BNUMD ROSS Ultra pH/ATC Triode) connected to a pH meter (Radiometer PHM 84, Copenhagen, Denmark or Orion Star A211 pH meter, ThermoFisher Scientific, Waltham, MA, USA).

Titrateable acid was measured by double endpoint titration as described by Brix and Grosell, (2013). Titration acid (0.01 N HCl) and base (0.01 N NaOH) were dispensed using 2 ml Gilson microburettes. Net titrateable acid and ammonia transport were calculated as described by Brix and Grosell, (2013).

All values are expressed as means $\pm$ SEM throughout. Series 1 kinetic data was observed to fit a Michaelis-Menten function and estimates of K_m and V_{max} were determined using non-linear regression. Comparison data for pharmacological inhibitor experiments in Series 1 and all Series 2 experiments were analyzed by Welch's *t*-test or when multiple treatments were evaluated, data were analyzed by ANOVA followed by Dunnett's post hoc test or if the data did not meet equal variance assumptions a Kruskal-Wallis test followed by Dunn's multiple comparison test was used ($P < 0.05$). All statistical analyses were performed using GraphPad Prism (v.5).

3. Results

3.1. Series 1: characterization of Na^+ uptake

3.1.1. Na^+ uptake kinetics

Sodium uptake rates increased with increasing ambient Na^+ concentrations, following a hyperbolic curve that approximated Michaelis-Menten saturation kinetics. The K_m and V_{max} were estimated using non-linear regression ($r^2 = 0.948$) and were $43 \pm 10 \mu\text{mol l}^{-1}$ and $204 \pm 12 \text{ nmol g}^{-1} \text{ h}^{-1}$, respectively (Fig. 1).

3.1.2. Pharmacological inhibitor experiments

Exposure to phenamil (Na^+ channel blocker) resulted in no significant inhibition of Na^+ uptake (Fig. 2A); exposure to EIPA (NHE

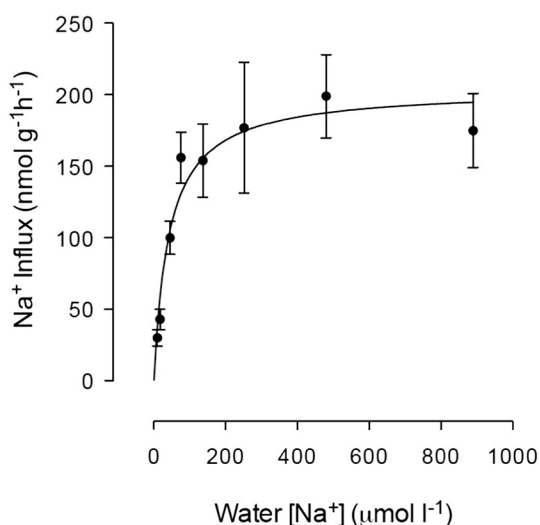


Fig. 1. Na^+ uptake rate ($\text{nmol g}^{-1} \text{ h}^{-1}$) as a function of external Na^+ concentration ($\mu\text{mol l}^{-1}$) for white sturgeon (*Acipenser transmontanus*) acclimated to $100 \mu\text{mol l}^{-1}$ Na^+ . Values are mean $\pm$ SEM ($n = 5$). Kinetic data were observed to fit a Michaelis-Menten function ($r^2 = 0.948$) and the Michaelis constant (K_m) and maximum transport velocity (V_{max}) were estimated to be $43 \pm 10 \mu\text{mol l}^{-1}$ and $204 \pm 12 \text{ nmol g}^{-1} \text{ h}^{-1}$, respectively.

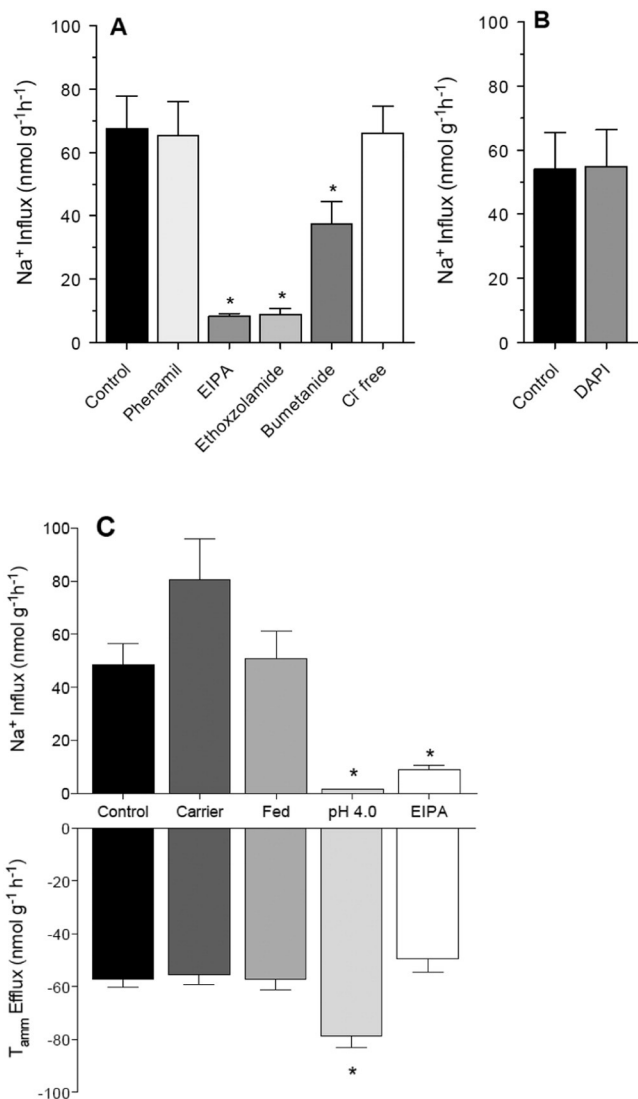


Fig. 2. Effect of different treatments on Na^+ uptake rate ($\text{nmol g}^{-1} \text{ h}^{-1}$) in white sturgeon (*Acipenser transmontanus*) acclimated $100 \mu\text{mol l}^{-1}$ Na^+ using phenamil (Na^+ channel inhibitor), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; NHE inhibitor), ethoxzolamide (CA inhibitor), bumetanide (NKCC inhibitor), Cl^- free water (Na_2SO_4) (A) and 4',6-diamidino-2-phenylindole (DAPI; acid sensing ion channel [ASIC] inhibitor) (B); concurrent Na^+ uptake and T_{amm} excretion rate ($\text{nmol g}^{-1} \text{ h}^{-1}$) measurements during exposure to each treatment (C). See methods and materials for a complete description of each treatment. Data are mean $\pm$ SEM ($n = 8$); difference compared to control (or carrier control – EIPA treatment in panel C only), significance indicated by asterisk ($P < 0.05$).

inhibitor) and ethoxzolamide (CA inhibitor) induced significant reductions in Na^+ uptake of 88% and 87%, respectively ($P < 0.01$; Fig. 2A). To test whether Na^+ uptake was chloride dependent, Na^+ uptake was measured following exposure to bumetanide (NKCC inhibitor), which significantly reduced Na^+ uptake by 45% ($P < 0.05$), or to Na_2SO_4 (Cl^- free water), which did not affect Na^+ uptake (Fig. 2A). Exposure to DAPI (ASIC inhibitor) resulted in no change in Na^+ uptake (Fig. 2B).

To determine if there is a relationship between NHE and Rh proteins, measurements of Na^+ uptake and T_{amm} efflux were taken following feeding, exposure to EIPA or pH 4.0 (Fig. 2C). There was no change in Na^+ uptake or T_{amm} excretion following feeding. EIPA exposure significantly reduced Na^+ uptake by 81% ($P < 0.01$), but T_{amm} efflux was unchanged. When fish were acclimated to 4.0 pH water, Na^+ uptake was nearly completely inhibited (97%) ($P < 0.001$) while T_{amm} efflux significantly increased by 39% ($P < 0.01$; Fig. 2C).

3.2. Series 2: acid-base disturbances, Na^+ uptake and net acid flux

3.2.1. Effect of hypercarbia on extracellular pH

Juvenile white sturgeon exposed to 1.5 or 6 kPa PCO_2 for 3 h experienced a reduction in pH_e . After 24 h exposure, pH_e nearly fully recovered at 1.5 kPa PCO_2 , whereas at 6 kPa PCO_2 , pH_e declined further ($P < 0.001$; Fig. 3). These results are consistent with those observed in adult white sturgeon (Baker et al., 2009).

3.2.2. Na^+ uptake during acid-base challenges

Hypercarbia exposure for 3 h resulted in a progressive reduction in Na^+ uptake as PCO_2 tension increased. At 3 and 6 kPa PCO_2 , Na^+ uptake was significantly reduced by 87 and 94% (Kruskal-Wallis test, $P < 0.001$), respectively. Exposure to low water pH in the absence of CO_2 reduced Na^+ uptake at water pH 5.2 (Kruskal-Wallis test, $P < 0.001$); there was no change at 5.9 or 5.5 compared to control. Na^+ uptake rates between equivalent water pH values in the presence and absence of CO_2 differ at each water pH ($P < 0.001$; Fig. 4).

Sodium uptake rates in response to 3 h hyperoxia (which increases internal PCO_2 to approximately 1–1.5 kPa) were compared to control and 3 h 1.5 kPa PCO_2 Na^+ uptake rates. Hyperoxia did not significantly increase Na^+ uptake rate over control values, but they were elevated over that of 3 h 1.5 kPa PCO_2 exposure (Kruskal-Wallis test, $P < 0.001$; Fig. 5). Sodium uptake rates were also examined at 1.5 kPa PCO_2 over 12 h. Compared to control, Na^+ uptake rate was reduced at 3 h and remained unchanged at 6 and 12 h (Kruskal-Wallis test, $P < 0.001$; Fig. 5).

3.2.3. Net acid flux during hypercarbia and low water pH

During a 3 h hypercarbia exposure, there were no changes in titratable acid, ammonia or net acid flux compared to control; these effluxes were significantly different from zero (no net flux) (one-sample t -test, $\mu_0 = 0$, $P < 0.05$; Fig. 6). Low water pH of 5.9 for 3 h resulted in titratable acid and net acid effluxes that were significantly different from control ($P < 0.05$). The effluxes at water pH 5.9 and 5.2 were significantly different from zero for titratable acid and ammonia but not for net acid (one-sample t -test, $\mu_0 = 0$, $P < 0.05$; Fig. 6).

4. Discussion

White sturgeon have a low capacity, high affinity Na^+ transport system that uses a NHE driven by H^+ generated via CA-mediate CO_2

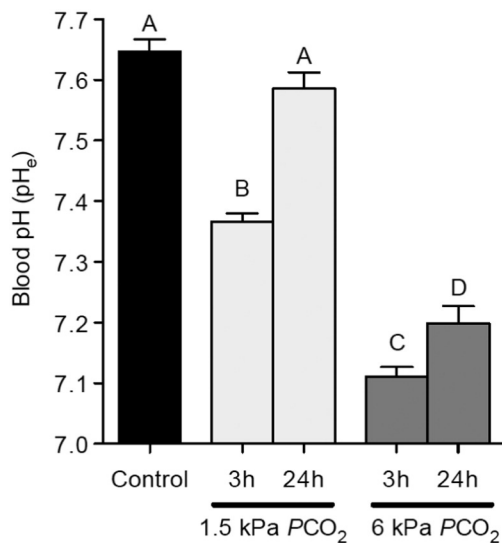


Fig. 3. Blood pH (pH_e) of white sturgeon during control CO_2 exposure (0.04 kPa PCO_2 ; black bar) or exposures to 1.5 kPa (light bars) or 6 kPa (dark bars) PCO_2 for 3 or 24 h. Data are mean $\pm$ SEM ($n = 8$); different letters indicate significant differences ($P < 0.05$).

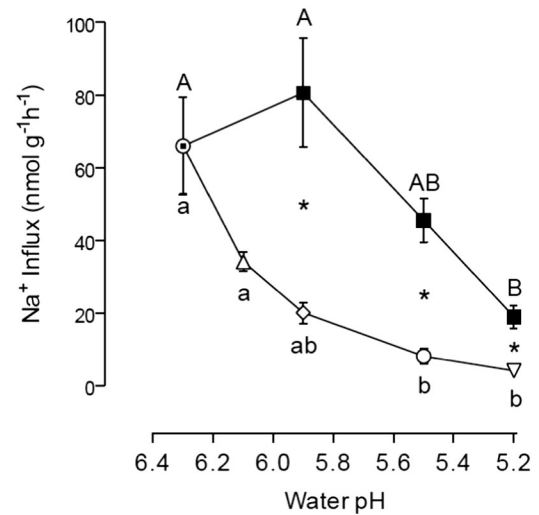


Fig. 4. Effect of acid-base disturbances on Na^+ uptake rates (nmol $\text{g}^{-1} \text{h}^{-1}$) in white sturgeon. Na^+ uptake rates were measured following 3 h exposure to control (○); air, 0.04, 0.75 (△), 1.5 (◇), 3 (○) or 6 (▽) kPa PCO_2 or to aerated water at 5.9, 5.5 or 5.2 pH units (■), which corresponded to water pH during 1.5, 3 and 6 kPa PCO_2 , respectively. In all treatments, ^{22}Na was added in the final hour of exposure for measurement of Na^+ uptake rate. Data are mean $\pm$ SEM ($n = 10$). Asterisks indicate significant differences between CO_2 and air exposures at same water pH; different uppercase letters denote significant differences between air exposures, different lowercase letters denote significant differences between CO_2 exposures ($P < 0.05$).

hydration as indicated in Series 1. Na^+ uptake results in Series 1 were consistent with our hypothesis of a low capacity, high affinity system for Na^+ uptake, but not with use of a hypothesized Na^+ channel or

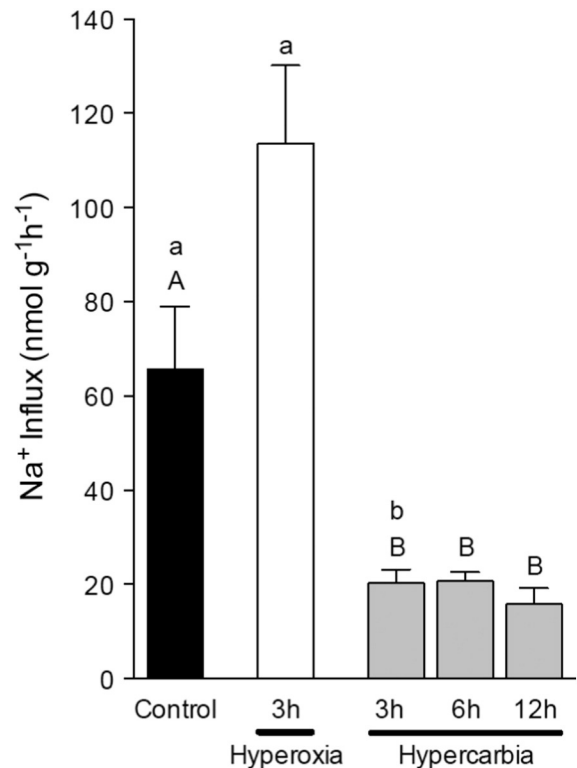


Fig. 5. Effect of 3 h exposure to hyperoxia (80 kPa PO_2 , 20 kPa PN_2) and exposure to 1.5 kPa PCO_2 for 3, 6 and 12 h on Na^+ uptake rate. In all treatments, ^{22}Na was added in the final hour of exposure for measurement of Na^+ uptake rate. Data are mean $\pm$ SEM. Different uppercase letters denote significant differences between control and hypercarbia, different lowercase letters denote significant differences between control and 3 h exposure to hyperoxia or hypercarbia ($P < 0.05$).

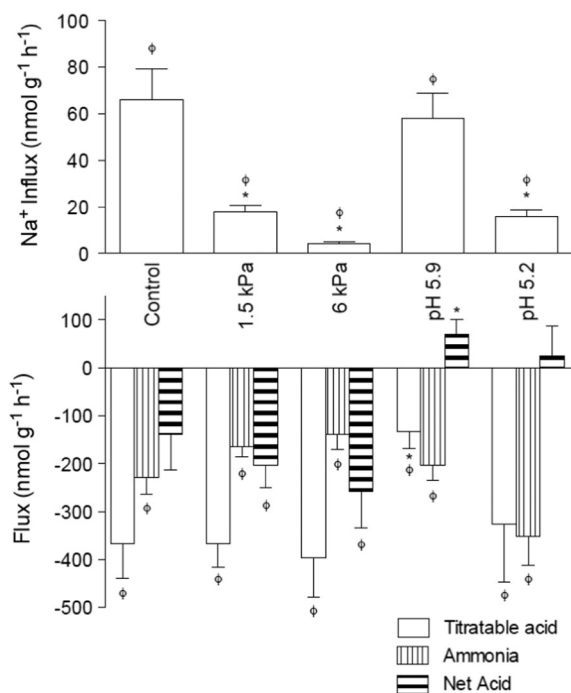


Fig. 6. Effect of hypercarbia and low water pH on Na⁺ uptake rate (nmol g⁻¹ h⁻¹) (data from Fig. 4), titratable acid, ammonia and net acid flux (nmol g⁻¹ h⁻¹). White sturgeon were exposed to either 1.5 or 6 kPa PCO₂, or water pH of 5.9 or 5.2 pH units for 3 h. Data are mean ± SEM (n = 6–8). Asterisks indicate significant difference compared to the control ($P < 0.05$); ϕ indicate significant difference from zero (no net flux) (one-sample t -test; $\mu_0 = 0$; $p < 0.05$).

ASIC/H⁺-ATPase for Na⁺ uptake. The effect of acid-base disturbances on Na⁺ uptake in Series 2 indicated that hypercarbia at all CO₂ tensions reduced Na⁺ uptake; this was inconsistent with the hypothesized increase in Na⁺ uptake at ≤1.5 kPa PCO₂ where pH_e recovery occurs. However, the reduction in Na⁺ uptake at higher PCO₂, where pH_e recovery does not occur, was in agreement with the hypothesis.

4.1. Characterization and mechanism of Na⁺ uptake in white sturgeon

Our objective in Series 1 was to characterize Na⁺ uptake in white sturgeon. We hypothesized that Na⁺ uptake in juvenile white sturgeon would be achieved by a low capacity, high affinity Na⁺ transporter system that is driven by a Na⁺ channel or ASIC/H⁺-ATPase. Characterization of white sturgeon Na⁺ uptake kinetics indicates a high Na⁺ affinity ($K_m = 43 \mu\text{mol l}^{-1}$) and a low capacity ($V_{max} = 204 \text{ nmol g}^{-1} \text{ h}^{-1}$) system for Na⁺ uptake, consistent with the first part of this hypothesis. This is the first time Na⁺ uptake kinetics have been measured in a basal actinopterygian and suggests that, at least in sturgeon, they are similar to that of freshwater teleosts inhabiting low Na⁺ water. For example, perch, trout, zebrafish and shiner have K_m s ranging from 21 to 158 $\mu\text{mol l}^{-1}$ (Boisen et al., 2003; Freda and McDonald, 1988); V_{max} was also similar to fishes of comparable size and at similar temperature [perch (249 nmol g⁻¹ h⁻¹), trout (379 nmol g⁻¹ h⁻¹) and shiner (460 nmol g⁻¹ h⁻¹) (Freda and McDonald, 1988)].

The lack of sensitivity to phenamil and DAPI in white sturgeon indicates they do not utilize an apical Na⁺ channel or ASIC/H⁺-ATPase system, which we hypothesized to drive Na⁺ uptake. Instead, they were very sensitive to EIPA which suggests an NHE is involved, similar to pupfish (*Cyprinodon* spp.) (Brix et al., 2015; Brix and Grosell, 2013), killifish (*Fundulus heteroclitus*) (Scott et al., 2005) and Osorezan dace (*Tribolodon hakonensis*) (Hirata et al., 2003). Due to the thermodynamically unfavorable environment for NHE function in low Na⁺ water (Parks et al., 2008), H⁺ are generated via CA-mediate CO₂ hydration

to facilitate NHE function as indicated by sensitivity of Na⁺ uptake to ethoxzolamide (Fig. 2A). Pupfish (Brix et al., 2015) and killifish (Scott et al., 2005) also appear to utilize CA generated H⁺ for the NHE function. In agreement with the use of CA, there appears to be no involvement of the NHE-Rh metabolon in white sturgeon due to the lack of linkage between Na⁺ influx and T_{amm} efflux (Fig. 2C); this mechanism is important for facilitating NHE function in some freshwater teleosts [e.g. zebrafish (Kumai and Perry, 2011; Shih et al., 2012), rainbow trout (Zimmer et al., 2010), and larval medaka (Wu et al., 2010)].

The use of a NHE for Na⁺ uptake in white sturgeon, a basal actinopterygian, may be due to the retention of the NHE for Na⁺ uptake from the ancestors of more basal marine vertebrates, as this system is observed in hagfish (Clifford et al., 2015; Tresguerres et al., 2006) and elasmobranchs (Choe, 2005; Evans et al., 2005; Gilmour and Perry, 2009). Additionally, NHE use in white sturgeon may be associated with their capacity for euryhalinity later in life (Mojazi Amiri et al., 2009); it is suggested that the absence of an apical Na⁺ channel H⁺-ATPase system and use of an NHE is common to euryhaline fishes (Brix and Grosell, 2013). It remains unclear which of these factors, if any, selects for the use of NHE in white sturgeon and clearly other basal actinopterygians should be investigated to clarify this.

While a NHE associated with CA appears to be the primary mechanism by which Na⁺ uptake occurs in white sturgeon, our results indicate a possible, albeit limited, role for an apical NKCC co-transporter given the modest reduction in Na⁺ uptake following bumetanide exposure (Fig. 2A). These results are conflicting with those of the Cl⁻ free exposure as there was no change in Na⁺ uptake in that treatment. Similar to NHE, operation of NKCC in a low NaCl water like the one used in these experiments should not be possible without generation of a micro-environment where gradients differ from bulk solutions. Indeed, Brix and Grosell (2012) demonstrated the role of an apical NKCC in Na⁺ uptake in *Cyprinodon variegatus* only at relatively high (7000 $\mu\text{mol l}^{-1}$) Na⁺ concentrations. Given the unfavorable thermodynamics and lack of support for a role of NKCC in the Cl⁻ free experiment, further studies are required to determine the significance of NKCC to Na⁺ uptake in white sturgeon.

4.2. Effect of respiratory acidosis on Na⁺ uptake

White sturgeon are exceptionally CO₂ tolerant and able to preferentially regulate pH_i regardless of pH_e recovery at all CO₂ tensions examined to date (Brauner and Baker, 2009; Shartau et al., 2016). However, at lower CO₂ tensions (1.5 kPa PCO₂) they also recover pH_e, but this does not occur at higher CO₂ tensions (6 kPa PCO₂) (Fig. 3). Increases in PCO₂ of around 1 kPa have been demonstrated to be associated with increased Na⁺ uptake and/or net plasma [Na⁺] in a number of aquatic animals [brown bullhead (Goss et al., 1992); trout (Wood et al., 1984); blue crab and channel catfish; (Cameron and Iwama, 1987); and Japanese flounder, yellowtail and dogfish (Hayashi et al., 2004)]. This increase in Na⁺ uptake is likely associated with net H⁺ efflux to enable these animals to compensate for respiratory acidosis. We hypothesized white sturgeon pH_e compensation would be associated with an increase in Na⁺ uptake during lower PCO₂ tension (≤1.5 kPa PCO₂). Series 2 results did not support our hypothesis as Na⁺ uptake was reduced by 49 and 69% (not statistically significant) during 3 h exposure at 0.75 and 1.5 kPa PCO₂, respectively (Fig. 4). Similarly, there was a significant reduction of 69–76% in Na⁺ uptake that persisted over 12 h at 1.5 kPa PCO₂ (Fig. 5), during which time pH_e recovery is occurring (Baker et al., 2009), and suggests that uptake of Na⁺ may not be important for pH_e recovery during hypercarbia. Furthermore, Na⁺ uptake does not appear to be linked to metabolic rate during hypercarbia in white sturgeon as Na⁺ uptake decreased by 87 and 94% at 3 and 6 kPa PCO₂, respectively (Fig. 4), yet metabolic rate increased by ~35% at 4 kPa PCO₂, but decreased by ~27% at 6 kPa PCO₂ (Baker and Brauner, 2012).

There appears to be a direct effect of hypercarbia on the inhibition of Na⁺ uptake as acidified water alone did not reduce Na⁺ uptake to the

degree that hypercarbia did (Fig. 4). This may be due to the speed at which CO₂ moves across the gills relative to H⁺ and thus, even though water pH was similar, hypercarbia exposed fish may have experienced a more rapid acidosis; consequently, the cytosol and extracellular compartment may have been rapidly acidified, thus affecting both intra- and extra-cellular components of Na⁺ transporters on the apical and basolateral membranes. Interestingly, a respiratory acidosis induced via hyperoxia (where water pH was not reduced) resulted in a trend toward an increase in Na⁺ uptake (Fig. 5) that was presumably associated with pH_e recovery, implying that water pH may influence the mechanism of pH_e compensation. Reductions in water pH, either via hypercarbia or changes to water chemistry, likely create an unfavorable gradient for NHE function. The results in white sturgeon are in contrast to what was observed in brown bullhead (Goss et al., 1992), where Na⁺ uptake increased during 1 kPa PCO₂; however, the initial water pH was ~8.3 which is higher than in this study (~6.3) and further indicates the influence that water chemistry may have. Similarity, the low pH of Vancouver tap water has been previously suggested to interfere with Na⁺ uptake in rainbow trout (Wood et al., 1984).

Despite Na⁺ uptake being reduced during hypercarbia and low water pH exposure, there was no significant reduction in the net acid efflux in three of the four treatments, further supporting the lack of coupling between H⁺ and Na⁺ exchange under these conditions (Fig. 6). As H⁺ efflux still occurred, it is unlikely to be associated with Na⁺/H⁺ exchange but instead may be associated with Cl[−]/HCO₃[−] exchange; thus, measuring Cl[−] flux in future studies would be valuable. The lack of detectable difference between control and hypercarbia/low water pH net acid flux may be a result of the relatively small change in H⁺ flux needed during acid-base regulation. Similarly, Perry et al. (1987) were unable to detect an increase in the efflux of acid equivalents when trout were exposed to 1 kPa PCO₂ for 72 h using a similar titration methodology; they calculated that the respiratory acidosis should have increased net acid excretion to approximately 55 nmol g^{−1} h^{−1} over the hypercarbia exposure. If similar (or slightly higher), acid efflux rates occur in sturgeon, it would be challenging to detect the small increase in efflux over background rates.

In conclusion, this is the first study, to our knowledge, to characterize Na⁺ uptake in a basal actinopterygian. We demonstrate that white sturgeon are unremarkable with respects to Na⁺ uptake kinetics and that they appear to rely on NHE for Na⁺ uptake that is driven by H⁺ generated via CA catalyzed hydration of CO₂; this is a similar strategy of Na⁺ uptake observed in some teleost fishes. The putative use of a NHE-CA system does not appear to be associated with pH_e compensation in white sturgeon as Na⁺ uptake was not associated with net H⁺ flux. Interestingly, imposing a concurrent external and internal acidosis via hypercarbia had a greater inhibitory effect on Na⁺ uptake than an external or internal acidosis alone – the reasons underlying these differences are presently unknown. Further studies are required to understand the mechanisms of acid-base regulation in sturgeon; as well, basal actinopterygians should be more broadly examined to gain insight into the evolution of ion and acid-base regulation in this group of vertebrates.

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