Comparative Biochemistry and Physiology, Part A 204 (2017) 197–204

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

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

Ryan B. Shartau *, Kevin V. Brix [1], Colin J. Brauner

*Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada*

ARTICLE INFO

*Article history:*
Received 20 September 2016
Received in revised form 1 December 2016
Accepted 2 December 2016
Available online 05 December 2016

*Keywords:*
*Acipenser transmontanus*
$Na^+$ uptake
Acid-base regulation
Ionoregulation
Hypercarbia
Fish physiology

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 $PCO_2$ but not at higher $PCO_2$ (6 kPa $PCO_2$) where they preferentially regulate intracellular pH ($pH_i$). It was hypothesized that during exposure to hypercarbia $Na^+$ uptake would increase at $CO_2$ 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 $PCO_2$, but at 6 kPa $PCO_2$ $Na^+$ uptake was reduced by 95% while low water pH equivalent to 6 kPa $PCO_2$ 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.



## 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 $Na^+/NH_4^+$ or $H^+ + NH_3$) 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 $HCO_3^-$ from metabolically produced $CO_2$, and $HCO_3^-$ 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, $Na^+$, $HCO_3^-$ co-transporter; NHE, $Na^+/H^+$ exchanger; Rh, Rhesus protein; $PCO_2$, partial pressure of $CO_2$; $pH_e$, extracellular pH; $pH_i$, intracellular tissue pH; $PO_2$, partial pressure of $O_2$.

* Corresponding author.
*E-mail address:* shartau@zoology.ubc.ca (R.B. Shartau).
[1] Present address: University of Miami, RSMAS, Miami, Florida, USA.

http://dx.doi.org/10.1016/j.cbpa.2016.12.004
1095-6433/

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 $<80$ μmol $l^{-1}$. 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 $PCO_2$ (hypercarbia) 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 acidoses induced by hypercarbia have been demonstrated to increase $Na^+$ uptake in some fish, likely in association with increased $H^+$ efflux and $pH_e$ compensation as observed in 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, hypercarbia exposure leads to increased extracellular [$Na^+$] in blue crab (*Callinectes sapidus*), channel catfish (*Ictalurus punctatus*) (Cameron and Iwama, 1987), Japanese flounder (*Paralichthys olivaceus*), yellowtail (*Seriola quinqueradiata*) and starspotted 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 hypercarbia 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 hypercarbia. We hypothesized that $Na^+$ uptake would increase during hypercarbia 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 $PCO_2$) hypercarbia 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 hypercarbia 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 hypercarbia lowers water pH, we wanted to distinguish between the effect of low water pH and hypercarbia 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 (hypercarbia) 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 hypercarbia and low water pH to investigate the relationship between $Na^+$ uptake and net acid flux during these acidoses.

## 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 μmol $l^{-1}$ $Na^+$, 69 μmol $l^{-1}$ $Cl^-$ (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 μmol $l^{-1}$ $Na^+$, 73 μmol $l^{-1}$ $Cl^-$, 7 μmol $l^{-1}$ $Mg^{2+}$, 89 μmol $l^{-1}$ $Ca^{2+}$ (Metro Vancouver, 2015), pH 6.3, 3 μmol $l^{-1}$ $HCO_3^-$] 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 μmol $l^{-1}$ $Na^+$. At each $Na^+$ concentration five fish were placed in 1000 ml of a defined medium (480 μmol $l^{-1}$ $CaSO_4$, 150 μmol $l^{-1}$ $MgSO_4$, 100 μmol $l^{-1}$ $KHCO_3$, 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 [$Na^+$] (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 [$Na^+$] 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 $^{22}Na$, then euthanized in a 100 mmol $l^{-1}$ $Na^+$ solution containing MS-222 (0.5 g $l^{-1}$ MS-222 buffered with 1 g $l^{-1}$ $NaHCO_3$) 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 $2 \times 10^{-5}$ mol $l^{-1}$ and 0.1% DMSO. Five minutes following inhibitor/DMSO addition, 1 μCi of $^{22}Na$ 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 $^{22}Na$ 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 $4 \times 10^{-5}$ 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 cotransporter (NKCC) inhibitor] and in a separate experiment to $Cl^-$-free media (50 μmol $l^{-1}$ $Na_2SO_4$ 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-sulfon-amide), 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 $HNO_3$); (iii) EIPA – fish were exposed to $5 \times 10^{-5}$ 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 $^{22}Na$ was added to the solution. The flux solution was sampled after 1 min for initial measurements of [$Na^+$] (2 ml), $^{22}Na$ 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 [$Na^+$], $^{22}Na$ 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 $PCO_2$; control), 1.5 or 6 kPa $PCO_2$; $PO_2$ was maintained at 21 kPa, balanced with $N_2$. 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 $NaHCO_3$ 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 $CO_2$, $O_2$ and $N_2$, 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 acidoses. To achieve an external acidosis, water pH was reduced with and without elevated $CO_2$ (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 $PO_2$ was maintained at 21 kPa. $Na^+$ uptake was measured by adding 1 μCi of $^{22}Na$ in the final hour of $CO_2$ 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 $CO_2$ tensions, fish were exposed to air (control), 0.75, 1.5, 3 or 6 kPa $PCO_2$; to investigate if $Na^+$ uptake changes during $pH_e$ recovery, fish were exposed to 1.5 kPa $PCO_2$ for 6 or 12 h. The effect of reduced water pH on $Na^+$ uptake in the absence of $CO_2$ was measured in fish exposed to a water pH of 5.9, 5.5 or 5.2, which was adjusted through the addition of $H_2SO_4$. These water pH values correspond with those measured during exposure to 1.5, 3 and 6 kPa $PCO_2$, respectively. Finally, we wanted to assess if $Na^+$ uptake varied when the source of $CO_2$ 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 $PO_2$ and 20 kPa $PN_2$), which creates a respiratory acidosis approximately equivalent to 1–1.5 kPa $PCO_2$ (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 $PCO_2$, or water pH of 5.9 and 5.2, which correspond with those measured during exposure to 1.5 and 6 kPa $PCO_2$, 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 $^{22}Na$

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).

Titratable 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 titratable acid and ammonia transport were calculated as described by Brix and Grosell, (2013).

All values are expressed as means ± 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 ± 10 μmol $l^{-1}$ and 204 ± 12 nmol $g^{-1}$ $h^{-1}$, 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 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).

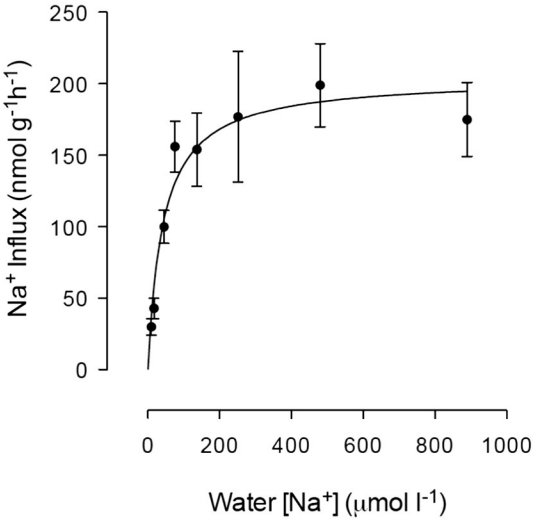


**Fig. 1.** $Na^+$ uptake rate (nmol $g^{-1}$ $h^{-1}$) as a function of external $Na^+$ concentration (μmol $l^{-1}$) for white sturgeon (*Acipenser transmontanus*) acclimated to 100 μmol $l^{-1}$ $Na^+$. Values are mean ± SEM (n = 5). Kinetic data were observed to fit a Michaelis-Menten function ($r^2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

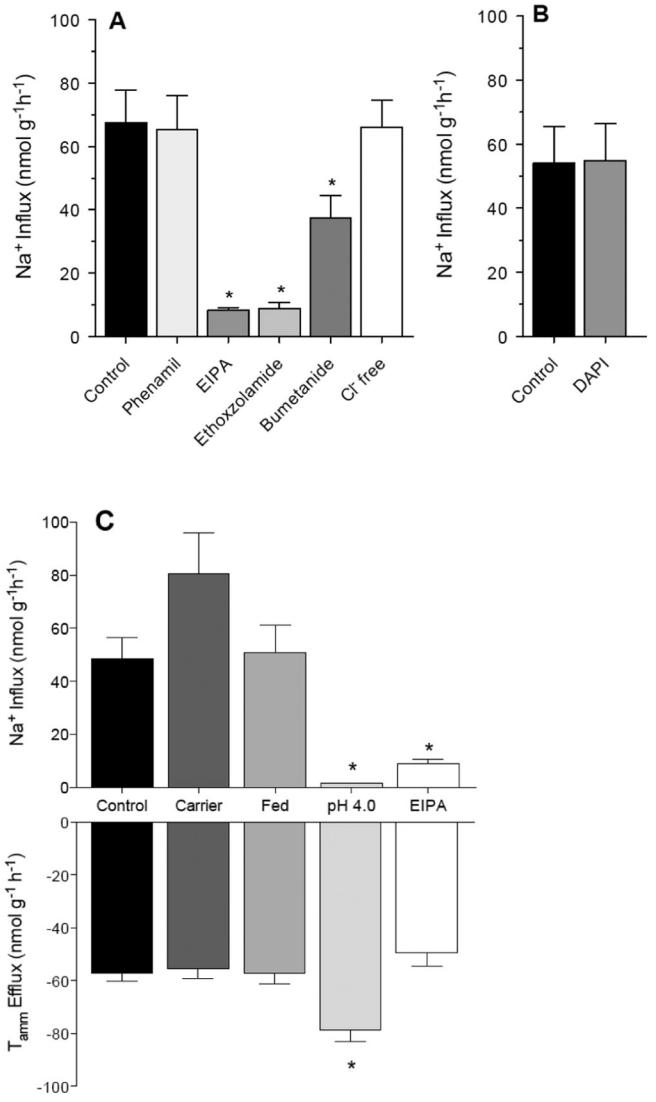


**Fig. 2.** Effect of different treatments on $Na^+$ uptake rate (nmol $g^{-1}$ $h^{-1}$) in white sturgeon (*Acipenser transmontanus*) acclimated 100 μmol $l^{-1}$ $Na^+$ using phenamil ($Na^+$ channel inhibitor), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; NHE inhibitor), ethoxzolamide (CA inhibitor), 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 (nmol $g^{-1}$ $h^{-1}$) measurements during exposure to each treatment (C). See methods and materials for a complete description of each treatment. Data are mean ± SEM (n = 8); difference compared to control (or carrier control – EIPA treatment in panel C only), significance indicated by asterisk ($P < 0.05$).

### 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 $CO_2$ 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

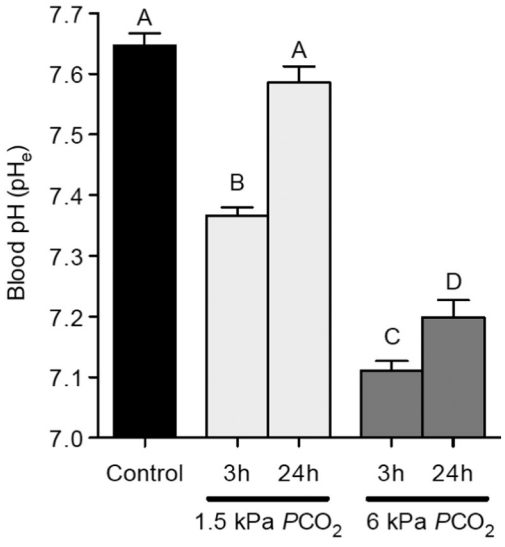


**Fig. 3.** Blood pH ($pH_e$) of white sturgeon during control $CO_2$ exposure (0.04 kPa $PCO_2$; black bar) or exposures to 1.5 kPa (light bars) or 6 kPa (dark bars) $PCO_2$ for 3 or 24 h. Data are mean ± SEM (n = 8); different letters indicate significant differences ($P < 0.05$).

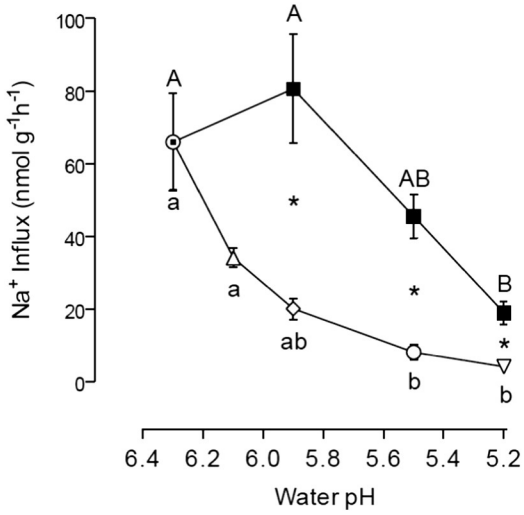


**Fig. 4.** Effect of acid-base disturbances on $Na^+$ uptake rates (nmol $g^{-1}$ $h^{-1}$) in white sturgeon. $Na^+$ uptake rates were measured following 3 h exposure to control (○; air, 0.04), 0.75 (△), 1.5 (◇), 3 (○) or 6 (▽) kPa $PCO_2$ or to aerated water at 5.9, 5.5 or 5.2 pH units (■), which 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 ± SEM (n = 10). Asterisks indicate significant differences between $CO_2$ and air exposures at same water pH; different uppercase letter denote significant differences between air exposures, different lowercase letters denote significant differences between $CO_2$ exposures ($P < 0.05$).

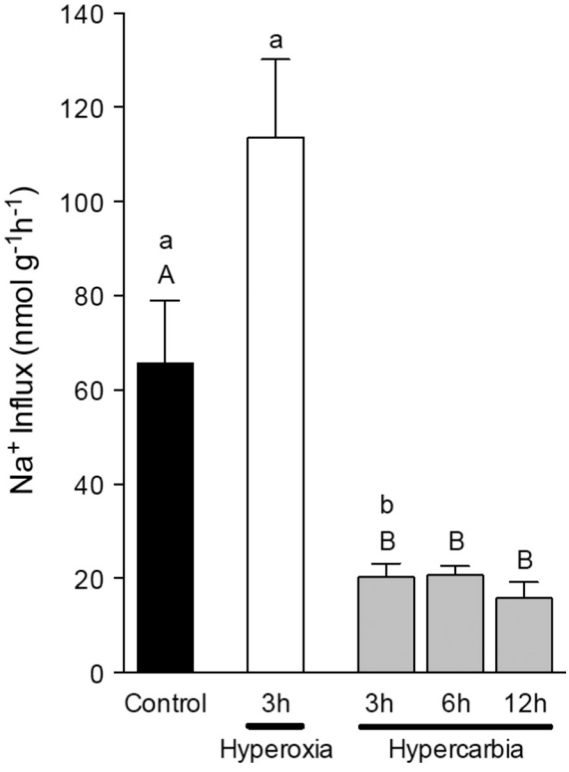


**Fig. 5.** Effect of 3 h exposure to hyperoxia (80 kPa $PO_2$, 20 kPa $PN_2$) and exposure to 1.5 kPa $PCO_2$ for 3, 6 and 12 h on $Na^+$ uptake rate. In all treatments, $^{22}Na$ was added in the final hour of exposure for measurement of $Na^+$ uptake rate. Data are mean ± SEM. Different uppercase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

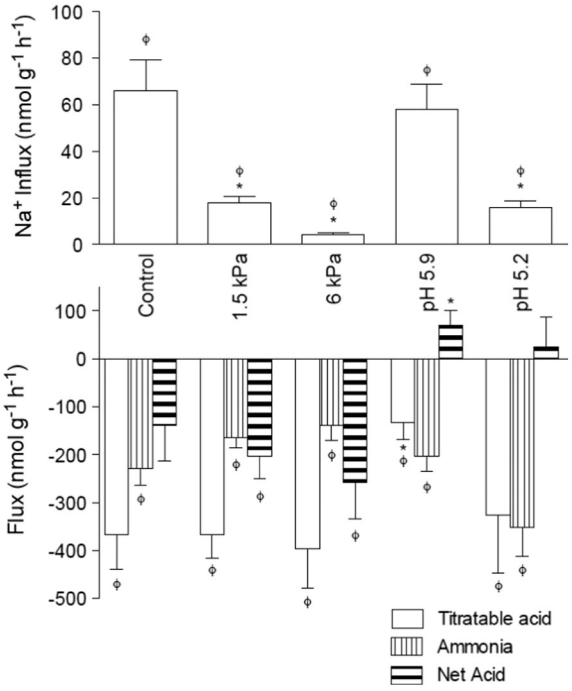


**Fig. 6.** Effect of hypercarbia and low water pH on $Na^+$ uptake rate (nmol $g^{-1}$ $h^{-1}$) (data from Fig. 4), titratable acid, ammonia and net acid flux (nmol $g^{-1}$ $h^{-1}$). White sturgeon were exposed to either 1.5 or 6 kPa $PCO_2$, or water pH of 5.9 or 5.2 pH units for 3 h. Data are mean ± 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 $CO_2$ tensions reduced $Na^+$ uptake; this was inconsistent with the hypothesized increase in $Na^+$ uptake at ≤1.5 kPa $PCO_2$ where $pH_e$ recovery occurs. However, the reduction in $Na^+$ uptake at higher $PCO_2$, 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 μmol $l^{-1}$) and a low capacity ($V_{max}$ = 204 nmol $g^{-1}$ $h^{-1}$) 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 μmol $l^{-1}$ (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 $CO_2$ 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 μmol $l^{-1}$) $Na^+$ 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 $CO_2$ tolerant and able to preferentially regulate $pH_i$ regardless of $pH_e$ recovery at all $CO_2$ tensions examined to date (Brauner and Baker, 2009; Shartau et al., 2016). However, at lower $CO_2$ tensions (1.5 kPa $PCO_2$) they also recover $pH_e$, but this does not occur at higher $CO_2$ tensions (6 kPa $PCO_2$) (Fig. 3). Increases in $PCO_2$ 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 $PCO_2$ tension (≤1.5 kPa $PCO_2$). 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 $PCO_2$, respectively (Fig. 4). Similarly, there was a significant reduction of 69–76% in $Na^+$ uptake that persisted over 12 h at 1.5 kPa $PCO_2$ (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 $PCO_2$, respectively (Fig. 4), yet metabolic rate increased by ~35% at 4 kPa $PCO_2$, but decreased by ~27% at 6 kPa $PCO_2$ (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 $CO_2$ 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 $PCO_2$; 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 $Cl^-/HCO_3^-$ 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 $PCO_2$ 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 $CO_2$; 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.

## Funding

R.B.S. was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Graduate Scholarship. C.J.B was supported by a NSERC Discovery Grant (261924–13) and Accelerator Supplement (446005–13). K.V.B. was supported by a National Science Foundation Post-doctoral Fellowship (DBI-1306452).

## Acknowledgments

DW Baker and the International Center for Sturgeon Studies (ICSS) at Vancouver Island University for supplying the fish . We thank the two anonymous reviewers for their helpful comments.

## References

Allen, P.J., Mitchell, Z.A., DeVries, R.J., Aboagye, D.L., Ciaramella, M.A., Ramee, S.W., Stewart, H.A., Shartau, R.B., 2014. Salinity effects on Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchill, 1815) growth and osmoregulation. J. Appl. Ichthyol. 30:1229–1236. http://dx.doi.org/10.1111/jai.12542.

Baker, D.W., Brauner, C.J., 2012. Metabolic changes associated with acid–base regulation during hypercarbia in the CO2-tolerant chondrostean, white sturgeon (*Acipenser transmontanus*). Comp. Biochem. Physiol. A 161:61–68. http://dx.doi.org/10.1016/j.cbpa.2011.09.002.

Baker, D.W., Matey, V., Huynh, K.T., Wilson, J.M., Morgan, J.D., Brauner, C.J., 2009. Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296:R1868–R1880. http://dx.doi.org/10.1152/ajpregu.90767.2008.

Betancur-R, R., Broughton, R.E., Wiley, E.O., Carpenter, K., Lopez, J.A., Li, C., Holcroft, N.I., Arcila, D., Sanciangco, M., Cureton II, J.C., Zhang, F., Buser, T., Campbell, M.A., Ballesteros, J.A., Roa-Varon, A., Willis, S., Borden, W.C., Rowley, T., Reneau, P.C., Hough, D.J., Lu, G., Grande, T., Arratia, G., Orti, G., 2013. The tree of life and a new classification of bony fishes. PLoS Curr. 5. http://dx.doi.org/10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288.

Boisen, A., Amstrup, J., Novak, I., Grosell, M., 2003. Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). Biochim. Biophys. Acta 1618:207–218. http://dx.doi.org/10.1016/j.bbamem.2003.08.016.

Brauner, C.J., Baker, D.W., 2009. Patterns of acid-base regulation during exposure to hypercarbia in fish. In: Glass, M.L., Wood, S.C. (Eds.), Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects. Springer-Verlag, Berlin, Germany:pp. 43–63 http://dx.doi.org/10.1007/978-3-540-93985-6.

Brix, K.V., Grosell, M., 2012. Comparative characterization of $Na^+$ transport in *Cyprinodon variegatus variegatus* and *Cyprinodon variegatus hubbsi*: a model species complex for studying teleost invasion of freshwater. J. Exp. Biol. 215:1199–1209. http://dx.doi.org/10.1242/jeb.067496.

Brix, K.V., Grosell, M., 2013. Characterization of $Na^+$ uptake in the endangered desert pupfish, *Cyprinodon macularius* (Baird and Girard). Conserv. Physiol. 1 1-8. http://dx.doi.org/10.1093/conphys/cot005.

Brix, K.V., Esbaugh, A.J., Mager, E.M., Grosell, M., 2015. Comparative evaluation of $Na^+$ uptake in *Cyprinodon variegatus variegatus* (Lacepede) and *Cyprinodon variegatus hubbsi* (Carr) (Cyprinodontiformes, Teleostei): evaluation of NHE function in high and low $Na^+$ freshwater. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 185:115–124. http://dx.doi.org/10.1016/j.cbpa.2015.04.002.

Cameron, J.N., 1976. Branchial ion uptake in arctic grayling: resting values and effects of acid-base disturbance. J. Exp. Biol. 64, 711–725.

Cameron, J.N., Iwama, G.K., 1987. Compensation of progressive hypercapnia in channel catfish and blue crabs. J. Exp. Biol. 133, 183–197.

Choe, K.P., 2005. NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289: R1520–R1534. http://dx.doi.org/10.1152/ajpregu.00048.2005.

City of Nanaimo, 2015. Water Quality Report. See. http://www.nanaimo.ca/assets/Departments/Engineering~Public~Works/Water~Supply/Publications~and~Forms/AnnualWaterQualityReport2015.pdf.

City of Revelstoke, 2010. Annual Water Report. See. http://www.cityofrevelstoke.com/DocumentCenter/Home/View/329.

City of Trail, 2013. Water Treatment Plant Raw Water Full Spectrum Analysis. See. http://www.trail.ca/en/live/resources/2013-wtp-raw-water-full-spectrum-analysis.pdf.

Clifford, A.M., Goss, G.G., Roa, J.N., Tresguerres, M., 2015. Acid/base and ionic regulation in hagfish. In: Edwards, S.L., Goss, G.G. (Eds.), Hagfish Biology. CRC Press, Boca Raton, FL, pp. 277–298.

Dymowska, A.K., Schultz, A.G., Blair, S.D., Chamot, D., Goss, G.G., 2014. Acid-sensing ion channels are involved in epithelial $Na^+$ uptake in the rainbow trout *Oncorhynchus mykiss*. Am. J. Physiol. Cell Physiol. 307:C255–C265. http://dx.doi.org/10.1152/ajpcell.00398.2013.

Evans, D.H., 2011. Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. Acta Physiol. 202:349–359. http://dx.doi.org/10.1111/j.1748-1716.2010.02186.x.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85:97–177. http://dx.doi.org/10.1152/physrev.00050.2003.

Fitzgibbon, Q.P., Baudinette, R.V., Musgrove, R.J., Seymour, R.S., 2008. Routine metabolic rate of southern bluefin tuna (*Thunnus maccoyii*). Comp. Biochem. Physiol. 150: 231–238. http://dx.doi.org/10.1016/j.cbpa.2006.08.046.

Freda, J., McDonald, D.G., 1988. Physiological correlates of interspecific variation in acid tolerance in fish. J. Exp. Biol. 136, 243–258.

Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid-base regulation in fish. J. Exp. Biol. 212:1647–1661. http://dx.doi.org/10.1242/jeb.029181.

Gonzalez, R.J., McDonald, D.G., 1994. The relationship between oxygen uptake and ion loss in fish from diverse habitats. J. Exp. Biol. 190, 95–108.

Goss, G.G., Laurent, P., Perry, S.F., 1992. Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). Cell Tissue Res. 268, 539–552.

Hayashi, M., Kita, J., Ishimatsu, A., 2004. Acid-base responses to lethal aquatic hypercapnia in three marine fishes. Mar. Biol. 144:153–160. http://dx.doi.org/10.1007/s00227-003-1172-y.

Hedges, S.B., Kumar, S., 2009. The Timetree of Life. Oxford University Press.

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M.H., Romero, M.F., Hirose, S., 2003. Mechanism of acid

adaptation of a fish living in a pH 3.5 lake. Am. J. Physiol. Regul. Integr. Comp. Physiol. 284:R1199–R1212. http://dx.doi.org/10.1152/ajpregu.00267.2002.

Hiroi, J., McCormick, S.D., Ohtani-Kaneko, R., Kaneko, T., 2005. Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for $Na^+$/$K^+$-ATPase, $Na^+$/$K^+$/$2Cl^-$ cotransporter and CFTR anion channel. J. Exp. Biol. 208: 2023–2036. http://dx.doi.org/10.1242/jeb.01611.

Hwang, P.-P., Lee, T.-H., Lin, L.-Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301: R28–R47. http://dx.doi.org/10.1152/ajpregu.00047.2011.

Kleyman, T.R., Cragoe Jr., E.J., 1988. Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. 105:1–21. http://dx.doi.org/10.1007/BF01871102.

Kumai, Y., Perry, S.F., 2011. Ammonia excretion via Rhcg1 facilitates $Na^+$ uptake in larval zebrafish, *Danio rerio*, in acidic water. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301: R1517–R1528. http://dx.doi.org/10.1152/ajpregu.00282.2011.

Lin, H., Randall, D., 1995. Proton pumps in fish gills. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Fish Physiology Vol. 14. Academic Press, San Diego, CA, pp. 229–255.

Maetz, J., 1956. Les échanges de sodium chez le poisson *Carassius auratus* L. Action d"un inhibiteur de l"anhydrase carbonique. J. Physiol. Paris.

McEnroe, M., Cech Jr., J.J., 1985. Osmoregulation in juvenile and adult white sturgeon, *Acipenser transmontanus*. Environ. Biol. Fish 14, 23–30.

Metro Vancouver, 2015. Vancouver Water Utility Annual Report. See. http://vancouver.ca/files/cov/water-quality-report.pdf.

Mojazi Amiri, B., Baker, D.W., Morgan, J.D., Brauner, C.J., 2009. Size dependent early salinity tolerance in two sizes of juvenile white sturgeon, *Acipenser transmontanus*. Aquaculture 286:121–126. http://dx.doi.org/10.1016/j.aquaculture.2008.08.037.

Parks, S.K., Tresguerres, M., Goss, G.G., 2008. Theoretical considerations underlying $Na^+$ uptake mechanisms in freshwater fishes. Comp. Biochem. Physiol. C 148:411–418. http://dx.doi.org/10.1016/j.cbpc.2008.03.002.

Perry, S.F., Malone, S., Ewing, D., 1987. Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid–base status. Can. J. Zool.

Scott, G.R., Claiborne, J.B., Edwards, S.L., Schulte, P.M., Wood, C.M., 2005. Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J. Exp. Biol. 208:2719–2729. http://dx.doi.org/10.1242/jeb.01688.

Shartau, R.B., Baker, D.W., Crossley, D.A., Brauner, C.J., 2016. Preferential intracellular pH regulation: hypotheses and perspectives. J. Exp. Biol. 219:2235–2244. http://dx.doi.org/10.1242/jeb.126631.

Shih, T.-H., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Lin, L.-Y., 2012. Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302:R84–R93. http://dx.doi.org/10.1152/ajpregu.00318.2011.

Swain, L.G., Eng, P., Walton, D.G., Phippen, B., Lewis, H., Brown, S., Bamford, G., Newsom, D., Lundman, I., 1998. Water Quality Assessment and Objectives for the Fraser River From Hope to Sturgeon and Roberts Banks. See. http://www.dfo-mpo.gc.ca/Library/272539.pdf.

Tresguerres, M., Parks, S.K., Goss, G.G., 2006. V-$H^+$-ATPase, $Na^+$/$K^+$-ATPase and NHE2 immunoreactivity in the gill epithelium of the Pacific hagfish (*Epatretus stoutii*). Comp. Biochem. Physiol. A 145:312–321. http://dx.doi.org/10.1016/j.cbpa.2006.06.045.

Verdouw, H., Van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. 12:399–402. http://dx.doi.org/10.1016/0043-1354(78)90107-0.

Wood, C.M., LeMoigne, J., 1991. Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. Respir. Physiol. 86:91–113. http://dx.doi.org/10.1016/0034-5687(91)90042-H.

Wood, C.M., Wheatly, M.G., Hobe, H., 1984. The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. Branchial exchanges. Respir. Physiol. 55, 175–192.

Wu, S.-C., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Wen, Z.-H., Lin, C.-S., Lin, L.-Y., 2010. Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. Am. J. Physiol. Cell Physiol. 298:C237–C250. http://dx.doi.org/10.1152/ajpcell.00373.2009.

Yan, J.J., Chou, M.Y., Kaneko, T., Hwang, P.P., 2007. Gene expression of $Na^+$/$H^+$ exchanger in zebrafish $H^+$-ATPase-rich cells during acclimation to low-$Na^+$ and acidic environments. Am. J. Physiol. Cell Physiol. 293:C1814–C1823. http://dx.doi.org/10.1152/ajpcell.00358.2007.

Zimmer, A.M., Nawata, C.M., Wood, C.M., 2010. Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and $Na^+$ uptake in freshwater rainbow trout. J. Comp. Physiol. B. 180:1191–1204. http://dx.doi.org/10.1007/s00360-010-0488-4.