

RESEARCH ARTICLE

Interaction of osmoregulatory and acid–base compensation in white sturgeon (*Acipenser transmontanus*) during exposure to aquatic hypercarbia and elevated salinity

Ciaran A. Shaughnessy^{1,*}, Dan W. Baker², Colin J. Brauner³, John D. Morgan² and Jason S. Bystriansky¹**ABSTRACT**

Migratory fishes encounter a variety of environmental conditions, including changes in salinity, temperature and dissolved gases, and it is important to understand how these fishes are able to acclimate to multiple environmental stressors. The gill is the primary site of both acid–base balance and ion regulation in fishes. Many ion transport mechanisms involved with acid–base compensation are also required for the regulation of plasma Na^+ and Cl^- , the predominant extracellular ions, potentially resulting in a strong interaction between ionoregulation and acid–base regulation. The present study examined the physiological interaction of elevated dissolved CO_2 (an acid–base disturbance) on osmoregulation during seawater acclimation (an ionoregulatory disturbance) in juvenile white sturgeon (*Acipenser transmontanus*). Blood pH (pH_e), plasma $[\text{HCO}_3^-]$, $[\text{Na}^+]$, $[\text{Cl}^-]$ and osmolality, white muscle water content, and gill Na^+/K^+ -ATPase (NKA) and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC) abundance were examined over a 10 day seawater (SW) acclimation period under normocarbica (NCSW) or during prior and continued exposure to hypercarbia (HCSW), and compared with a normocarbica freshwater (NCFW) control. Hypercarbia induced a severe extracellular acidosis (from pH 7.65 to pH 7.2) in HCSW sturgeon, and these fish had a 2-fold greater rise in plasma osmolality over NCSW by day 2 of SW exposure. Interestingly, pH_e recovery in HCSW was associated more prominently with an elevation in plasma Na^+ prior to osmotic recovery and more prominently with a reduction in plasma Cl^- following osmotic recovery, indicating a biphasic response as the requirements of osmoregulation transitioned from ion-uptake to ion-excretion throughout SW acclimation. These results imply a prioritization of osmoregulatory recovery over acid–base recovery in this period of combined exposure to acid–base and ionoregulatory disturbances.

KEY WORDS: Osmoregulation, Acid–base balance, Seawater, Hypercarbia, Multiple stressors

INTRODUCTION

Although there is a great deal known about how fishes acclimate to environmental stressors in isolation (such as acclimating to changes in salinity: reviewed by Evans et al., 1999; Marshall and Grosell, 2005), much less is known about how fishes respond to multiple,

potentially interacting, environmental stressors. For instance, it is not well understood in any anadromous fishes how ion and water balance during salinity acclimation are affected by other environmental stressors likely encountered during a seaward migration (such as changes in temperature and dissolved gases). One such ‘second stressor’ likely to be encountered in this case is aquatic hypercarbia (elevated environmental P_{CO_2}) (Cech and Doroshov, 2005), which can occur regularly in highly productive freshwater and brackish environments as a result of night-time CO_2 production by aquatic flora and can be exacerbated by poor water mixing (such as in slow-moving rivers, etc.) or anthropogenic stimuli (Heisler, 1982; Ultsch, 1996; Burnett, 1997). Exposure of water-breathing fishes to aquatic hypercarbia can cause a reduction in blood pH (pH_e), which results in a respiratory acidosis (reviewed by Brauner and Baker, 2009). Characterizing how the seaward migration made by anadromous fishes is affected by hypercarbia may become even more relevant in the near future as anthropogenic P_{CO_2} in both marine (Caldeira and Wickett, 2003) and freshwater (Sayer et al., 1993) systems increase.

In freshwater (FW) fishes, Na^+ and Cl^- are actively taken up across the gill epithelium to counter the passive loss of these osmolytes to the more dilute environment. After entry into seawater (SW), a fish must increase its drinking rate to balance the osmotic loss of water to the more solute-concentrated environment and actively excrete Na^+ and Cl^- from the gill to maintain ionic and osmotic homeostasis. Thus, for a fish migrating from FW to SW, the gill epithelium must transform from a site of active ion uptake to a site active ion excretion (Evans et al., 1999; Marshall and Grosell, 2005). Movement of these ions across the gill epithelium is accomplished by secondary active transport processes which utilize the local ion concentration gradients maintained by the activity of the basolateral Na^+/K^+ -ATPase (NKA). In SW-acclimated fishes, Na^+ is excreted out of the gill via paracellular passage (facilitated by the transepithelial electrogenic gradients produced by the NKA) through ‘leaky’ junctions between gill epithelial cells, and Cl^- is excreted transcellularly via a basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC) and an apical chloride channel (CFTR) (Silva et al., 1977).

Generally, during exposure to aquatic hypercarbia, an organism undergoes an initial rapid respiratory acidosis, which is compensated for by a more gradual metabolic alkalosis and recovery of pH_e to normocapnic levels. The initial acidosis occurs as blood and tissue P_{CO_2} (P_{aCO_2}) equilibrate with the increase in environmental P_{CO_2} (P_{wCO_2}). In water-breathing fishes, recovery of pH_e is achieved primarily by transport of acid–base molecular equivalents across the gill epithelium through changes, for example, in proton (H^+) excretion and/or bicarbonate ion (HCO_3^-) uptake, generally in exchange with acid–base relevant ions, such as Na^+ and Cl^- , respectively. After dissociation of intracellular CO_2 in the presence of carbonic anhydrase (CA), H^+ may be excreted in

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exchange for Na^+ via an apical Na^+/H^+ exchanger (NHE) (primarily in marine-adapted fishes) or an apical H^+ -ATPase with an associated Na^+ channel (primarily in FW-adapted fishes), while HCO_3^- uptake may occur via either a basolateral $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger (AE1) or a basolateral $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBC) (for review, see Evans et al., 2005). Just as in salinity acclimation, these secondary active transport mechanisms associated with acid–base balance are also reliant on the electrochemical gradients produced by the NKA. Thus, the same gill epithelium that is responsible for osmoregulation during salinity acclimation is also the primary site of acid–base compensation during exposure to aquatic hypercarbia.

Consequently, it has been hypothesized that these two ionoregulatory processes (i.e. osmoregulation and acid–base balance at the gill) might interact during simultaneous respective challenges (Iwama and Heisler, 1991), although empirical support for this interaction is lacking. The white sturgeon (*Acipenser transmontanus* Richardson 1836) is a particularly interesting species in which to address this question because in addition to being one of the most basal extant ray-finned fishes, it is euryhaline (tolerant of a wide range of salinities), and is very tolerant of acid–base disturbances (Baker et al., 2009). White sturgeon juveniles rear in FW and may migrate to brackish waters and even full strength SW as adults (Wilson and McKinley, 2004). Despite their known ability to tolerate SW as adults, little is known about the development of hypo-osmoregulatory ability as juveniles. A study on Fraser River white sturgeon osmoregulation by Amiri et al. (2009) observed high mortality in 14 month old white sturgeon within 24 h of exposure to 16‰ salinity. It has also been shown that white sturgeon are very tolerant to severe aquatic hypercarbia (up to at least 6 kPa P_{wCO_2}) (Baker et al., 2009). At lower P_{wCO_2} tensions (1.5 kPa P_{wCO_2}), white sturgeon can effectively regulate pH_e , and at high (6 kPa) P_{wCO_2} tensions, they have an impressive ability to regulate intracellular pH (pH_i) in most tissues despite a large depression in pH_e , and thus are able to tolerate prolonged extracellular acidosis (pH_e reduced ~ 0.7 pH units) (Baker et al., 2009).

The primary hypotheses of this study were: (1) 2 year old white sturgeon possess the osmoregulatory capacity to acclimate to SW and (2) acidosis compensation and osmoregulatory compensation interact in the transport of Na^+ and Cl^- . To address these hypotheses, this study used salinity (20‰) and hypercarbia (6 kPa P_{wCO_2}) challenges that were within the known tolerance for white sturgeon but close to their upper tolerance limits. It was predicted that if acidosis compensation in SW is predominantly associated with Na^+/H^+ exchange, then the counter-productive transport of Na^+ (i.e. uptake for acidosis compensation versus excretion for osmoregulation) would limit one or both of these physiological processes (e.g. SW acclimation would be slowed in the presence of a respiratory acidosis). However, if acidosis compensation in SW is associated with HCO_3^- uptake in exchange for Cl^- excretion, the elimination of Cl^- required for both SW acclimation and acid–base compensation would accelerate one or both of these physiological processes.

Observing acid–base compensation in a fish acclimating to SW may reveal intricacies of the hypothesized interaction of physiological function regarding acid–base and osmoregulatory compensation that are undetectable in FW- or SW-adapted fish alone. Furthermore, investigating organismal and gill function adjustments to this multiple stressor scenario in a basal anadromous fish species will provide valuable information about the relationship between ionoregulatory and acid–base regulatory strategy, and provide an interesting evolutionary context for later comparisons with the more derived teleosts.

RESULTS

Fish transferred to SW in both normocarbia and hypercarbia remained active and appeared in good health throughout the 10 day experiment, with the exception of the hypercarbic SW group where eight individuals (of the initial 54 fish in the group) became moribund between days 2 and 4.

Ion and water balance

In the normocarbic freshwater (NCFW) control, plasma osmolality and ion (Na^+ and Cl^-) levels, as well as white muscle water content, remained stable throughout the 10 day experiment [mean levels pooled from all time points were as follows: osmolality = 252 ± 1 mOsmol kg^{-1} (Fig. 1A); $[\text{Na}^+] = 133 \pm 1$ mmol l^{-1} (Fig. 1B); $[\text{Cl}^-] = 120 \pm 1$ mmol l^{-1} (Fig. 1C); and white muscle water content = $79.4 \pm 0.2\%$ water (Fig. 1D)]. Within 6 h of SW exposure, plasma osmolality, $[\text{Na}^+]$ and $[\text{Cl}^-]$ in the normocarbic seawater (NCSW) sturgeon were significantly increased relative to NCFW levels and peaked at 24 h. However, after 4 days, plasma osmolality, $[\text{Na}^+]$ and $[\text{Cl}^-]$ had recovered to an approximate steady state and were no longer significantly different from NCFW levels. Changes in white muscle water content in NCSW sturgeon were consistent with changes in plasma ions, with maximal dehydration at 48 h, but values were also no longer significantly different after 4 days.

In hypercarbic seawater (HCSW), the time course of changes in plasma osmolality, $[\text{Na}^+]$ and $[\text{Cl}^-]$, as well as changes in white muscle water content, were similar to those observed in NCSW but were greater in magnitude and took longer to recover (Fig. 1). By 24 h after exposure to SW, when the magnitude of changes was greatest, HCSW plasma osmolality and $[\text{Na}^+]$ had increased by 30% and 35% over NCFW, respectively, before recovering by day 10. These changes were approximately 2-fold greater than the changes observed in NCSW. HCSW plasma $[\text{Cl}^-]$ exhibited a different pattern of change in that it did not increase above NCSW levels in the first 4 days, but significantly decreased between days 4 and 10. HCSW white muscle was maximally dehydrated (significantly below NCSW) at day 4, before recovering by day 7.

Acid–base balance and hematology

NCFW pH_e remained stable at pH 7.62 ± 0.01 (pooled value) (Fig. 2A). NCSW pH_e exhibited a transient but significant acidosis after transfer to SW, returning to NCFW values by day 4. This transient acidosis corresponded with a slight increase in plasma $[\text{HCO}_3^-]$ (Fig. 2B), but was not accompanied by any significant changes in blood oxygen carrying capacity as hematocrit (Hct), hemoglobin concentration ($[\text{Hb}]$) and mean corpuscular hemoglobin concentration (MCHC) (Table 1) remained relatively stable in all normocapnic sturgeon over the 10 day experiment. NCSW pH_i (Fig. 2C) was not different from NCFW at any sampling period despite the transient changes in pH_e .

After a 24 h hypercarbia exposure (i.e. on day=0 of SW exposure; see explanation in Materials and Methods section), HCSW sturgeon exhibited a significant extracellular acidosis. By day 1 of SW exposure, some compensation of pH_e had occurred and was associated with increasing plasma $[\text{HCO}_3^-]$; however, HCSW pH_e still remained significantly depressed compared with NCFW pH_e . Between 1 and 7 days after SW exposure, HCSW pH_e remained steady and significantly depressed compared with control values. By day 10, pH_e in HCSW had recovered to NCFW and NCSW values. HCSW white muscle pH_i did not change over the 10 day experiment despite the significant acidosis.

HCSW sturgeon did exhibit elevated Hct at day 0. However, this was in the absence of changes in blood $[\text{Hb}]$, which resulted

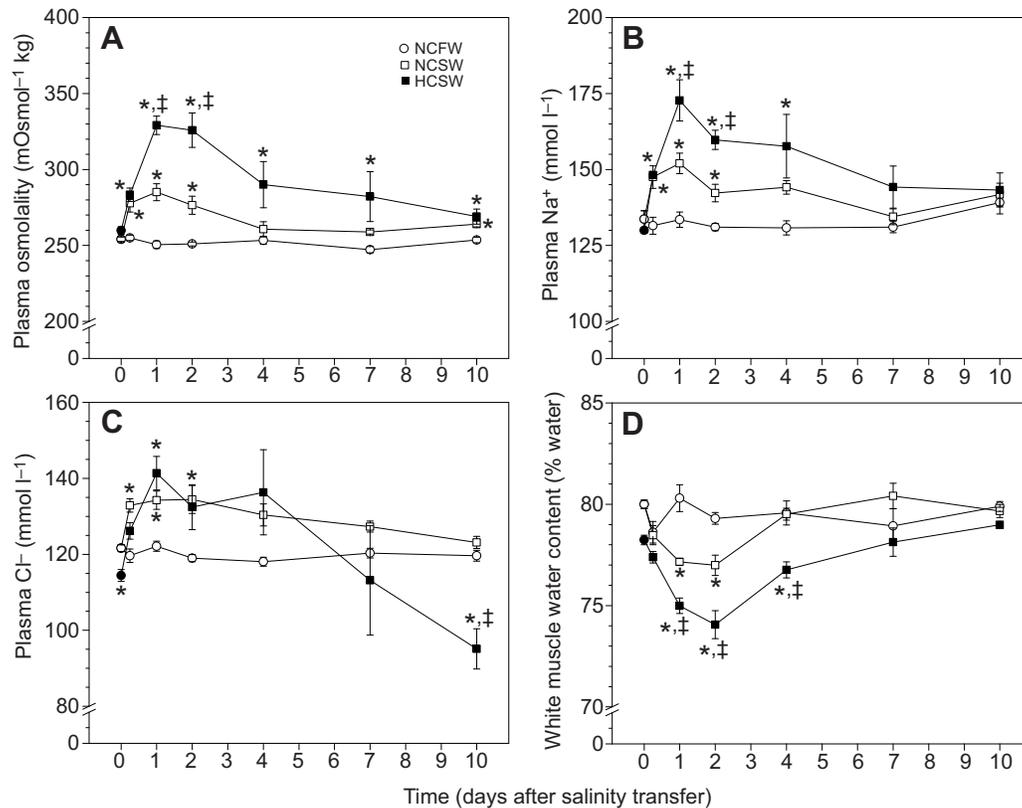


Fig. 1. Ion and water balance. Plasma osmolality (A), sodium (B) and chloride (C) concentration, and white muscle water content (D) in white sturgeon acclimated to normocarbic freshwater (NCFW), normocarbic seawater (NCSW) or hypercarbic seawater (HCSW) over 10 days. Values represent means \pm s.e.m. Symbols denote a statistically significant difference from NCFW (*) or between NCSW and HCSW (\ddagger) at the respective time point.

in significantly lower calculated values of MCHC. These changes were transient, and by day 10 there were no differences in Hct, [Hb] or MCHC values between experimental groups.

Gill protein activity and immunoblotting

Gill NKA activity was lowest in the NCFW control (compared with NCSW and HCSW) at every sampling period throughout the 10 day experiment (Fig. 3A). NCSW NKA activity increased 4-fold during the first 7 days, significantly above NCFW levels. HCSW NKA activity increased 6-fold during the first 7 days, significantly above NCFW but not NCSW levels.

Gill NKCC (225 kDa) abundance on day 4 was significantly greater (by over 2-fold) in the SW groups compared with the NCFW control, but NKCC abundance was not different between NCSW and HCSW sturgeon (Fig. 3B,C). Although only a slight increase in gill NKA α -subunit (110 kDa) abundance was observed in NCSW by day 4, there was a significant increase in NKA α -subunit abundance in HCSW compared with NCFW and NCSW (Fig. 3B,D). NKA α -subunit abundance in HCSW was nearly 3-fold that in NCFW, and 2-fold that in NCSW.

Plasma [Na⁺]-[Cl⁻] difference

The difference between plasma [Na⁺] and [Cl⁻] (Na-Cl; see description in Materials and methods) in NCFW and NCSW remained relatively stable at \sim 15 mmol l⁻¹ throughout the 10 day experiment (Fig. 4A). In HCSW, plasma Na-Cl rose significantly above NCFW and NCSW levels at two distinct times, on day 1 and again on day 10. Na-Cl in this group was significantly associated with plasma [HCO₃⁻] ($m=0.63$, $r^2=0.77$, $P<0.001$) (Fig. 4B).

DISCUSSION

NCSW acclimation in white sturgeon

This study is the first to describe the time course of osmotic compensation in elevated salinity by juvenile white sturgeon. The plasma osmolality of white sturgeon in the NCFW control presented here (252 ± 0.8 mOsmol kg⁻¹) is similar to reports from other studies on juvenile (Amiri et al., 2009) and adult (McEnroe and Cech, 1985) white sturgeon, indicating that 'normal' white sturgeon plasma osmolality levels are lower than levels observed in representative FW teleosts (\sim 275 mOsmol kg⁻¹) (Evans et al., 2005). When transferred to SW, fishes experience two major physiological disturbances: osmotic water loss and elevation of plasma ion concentrations. Similar to what is seen in other euryhaline fishes, white sturgeon plasma ion levels increased following exposure to NCSW (referred to as the 'crisis phase' of acclimation, characterized by increasing plasma ions as the fish transitions to hypo-osmoregulating) (Gordon, 1959), then were eventually returned to a new steady state nearer to FW control values. This rise and recovery of plasma ion concentrations coincided with a dehydration and rehydration of the white muscle within approximately 4 days. A 4 day adjustment period is common among anadromous fishes; similar (or shorter) timelines have been reported in other euryhaline fishes: chum salmon, 12 h (Black, 1951); killifish, 4–5 days (Marshall et al., 1999); rainbow trout, 4–5 days (Leray et al., 1981). In addition to the osmotic disturbances seen during SW acclimation, white sturgeon in this study also exhibited a transient change in pH_e. Reports on acid–base changes in response to elevated salinity have been variable [for instance, Perry and Heming (1981) reported an alkalosis during a SW transfer in rainbow trout]; however, most studies report an acidosis and subsequent recovery

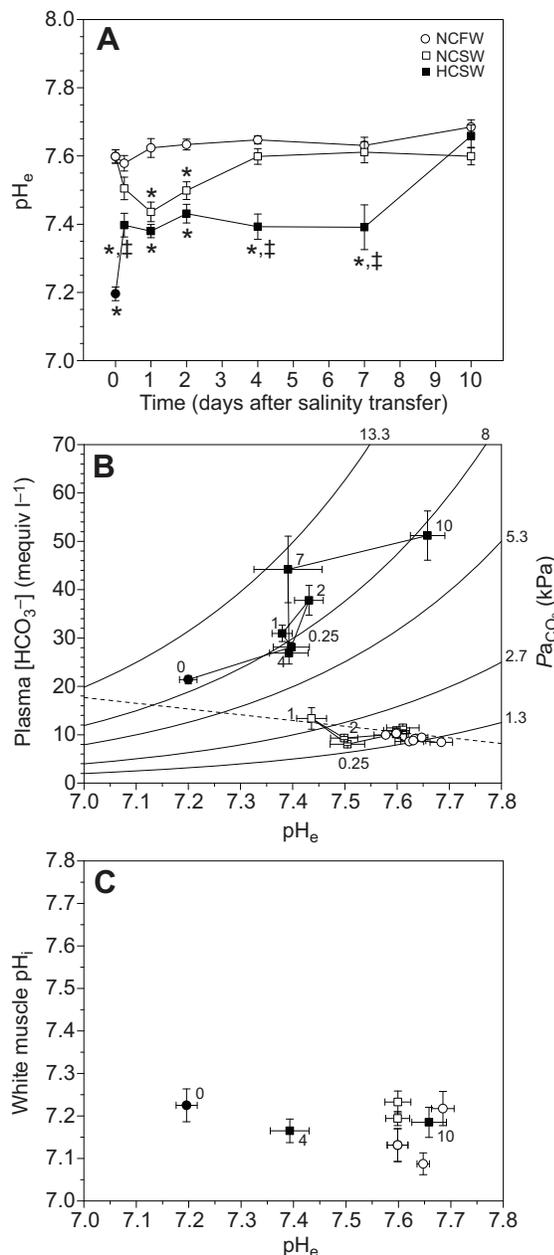


Fig. 2. Changes in acid–base status in white sturgeon acclimated to NCFW, NCSW or HCSW over 10 days. (A) Blood pH (pH_e). (B) Relationship between pH_e , HCO_3^- and blood and tissue P_{CO_2} (P_{aCO_2}). Curved lines represent P_{aCO_2} isopleths and the dashed line represents the blood non-bicarbonate buffer line (adapted from Baker et al., 2009). (C) Relationship between pH_e and white muscle intracellular pH (pH_i). Numbers on graphs indicate the day of SW acclimation for selected values. Values represent means \pm s.e.m. Symbols denote a statistically significant difference from NCFW (*) or between NCSW and HCSW (±) at the respective time point.

similar to the one observed in this study (Maxime et al., 1991; Larsen and Jensen, 1992; Madsen et al., 1996).

The recovery and stabilization of plasma ion concentrations in NCSW coincided with a significant increase in gill NKA activity and NKCC protein abundance, which is characteristic of euryhaline fishes acclimating to SW. It is interesting that the significant rise in NKA activity did not correspond with an increase in protein abundance. This may indicate a change in NKA isoform expression, similar to the α -subunit isoform switching commonly seen in

anadromous salmonids during SW acclimation (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky and Schulte, 2011), but this is yet to be examined in sturgeon. The response in gill NKA and NKCC observed in this study supports the conclusion that 2 year old white sturgeon are indeed capable of making the physiological adjustments necessary to enter brackish waters. These findings suggest that juvenile white sturgeon develop the ability to hypo-osmoregulate between 14 months (the oldest age they are known to lack SW tolerance; Amiri et al., 2009) and 24 months old (the age of the sturgeon in the present study).

The effect of hypercarbia on SW acclimation

The addition of aquatic hypercarbia as a second stressor appeared to extend the adjustment period during SW acclimation – the HCSW sturgeon acclimated to SW within a 4–7 day period (versus 4 days in NCSW). By day 7, all plasma ion parameters and white muscle water content in HCSW had returned to NCSW levels. However, during the crisis phase of acclimation, there were significantly higher maximum values of plasma $[Na^+]$ (but not $[Cl^-]$), osmolality and white muscle dehydration in HCSW than in NCSW. That white sturgeon had experienced greater white muscle dehydration in HCSW than NCSW, despite identical ambient salinity conditions, may suggest that the additional water leaving the white muscle in the HCSW sturgeon was associated with dilution of the blood plasma rather than an increased osmotic loss of water to the environment.

The 24 h exposure to aquatic hypercarbia induced an expected extracellular acidosis in the white sturgeon. As indicated by the pH_e – $[HCO_3^-]$ relationship (Fig. 2B), this initial acidosis occurred along a previously determined white sturgeon non-bicarbonate buffer line (Baker et al., 2009) as P_{aCO_2} equilibrated with P_{wCO_2} . Although exposure to SW appeared to transiently aid in pH_e recovery, the sturgeon remained in a severe acidosis for at least the first 7 days after salinity transfer. Their survival during such an acidosis may be unique to some of the more basal fishes, including sturgeon, which are able to regulate pH_i independently of pH_e , known as ‘preferential pH_i regulation’ (Brauner and Baker, 2009). Indeed, despite the large fluctuations in pH_e , white muscle pH_i in HCSW remained stable, indicating that the white sturgeon in the present study were exhibiting preferential pH_i regulation. A previous study on sturgeon (Baker et al., 2009) described a pH_e recovery from a lower P_{wCO_2} tension within 48 h, but no pH_e recovery within 48 h at P_{CO_2} tensions closer to that used in this study. Both the longer time frame of this study (10 days here, as opposed to 4 days in Baker et al., 2009) and the elevated salinity may have been factors in the eventual pH_e recovery (between 7 and 10 days) by the white sturgeon in HCSW; however, further studies are required to address this. Interestingly, these results may imply a prioritization of osmoregulatory recovery over acid–base recovery in this period of combined exposure to acid–base and ionoregulatory disturbances.

Gill NKCC protein abundance in HCSW was similar to that in NCSW. This is reflective of the identical osmoregulatory challenge (direct exposure to 20‰ salinity) that white sturgeon in each of these groups experienced. However, gill NKA activity and abundance were increased in response to the additional stress of aquatic hypercarbia. This may have two possible explanations. First, the greater NKA abundance in HCSW over NCSW may simply be associated with the higher plasma ion levels (although not ambient salinity) in HCSW. However, more likely, the greater NKA abundance in HCSW could be due to the additional acid–base balance challenge in this group. Much like in osmoregulation, during an acid–base disturbance, the NKA provides an electrochemical gradient that drives secondary ion transport, and is proposed to initiate acidosis compensation (Hirata

Table 1. Hematocrit, hemoglobin concentration and mean corpuscular hemoglobin concentration in white sturgeon acclimated to normocarbic freshwater (NCFW), normocarbic seawater (NCSW) or hypercarbic seawater (HCSW) over 10 days

	0 h	6 h	1 day	2 days	4 days	7 days	10 days
NCFW							
Hct	19.4±1.0	20.7±1.3	22.1±0.9	22.5±0.8	20.4±1.5	26.4±1.8	21.3±0.9
[Hb]	1.5±0.1	1.5±0.1	1.5±0.1	1.5±0.1	1.8±0.1	1.6±0.1	1.3±0.1
MCHC	7.6±0.6	7.73±0.4	7.1±0.3	6.6±0.5	9.0±1.2	6.0±0.3	6.4±0.3
NCSW							
Hct	–	24.9±0.5	26.4±1.0	22.5±0.7	17.8±1.2	19.1±1.8*	21.3±1.3
[Hb]	–	1.7±0.1	1.8±0.1	1.7±0.2	1.3±0.1	1.5±0.2	1.5±0.1
MCHC	–	6.9±0.2	6.7±0.3	7.5±0.7	7.2±0.5	8.2±1.1	7.5±0.7
HCSW							
Hct	35.4±1.4*	28.8±1.5*	31.0±2.3*	29.1±3.4	16.8±1.6	18.0±1.9*	19.1±3.5
[Hb]	1.6±0.1	1.6±0.1	1.8±0.1	3.4±0.9*‡	1.2±0.2*	1.3±0.1	1.4±0.1
MCHC	5.3±0.3*	5.6±0.5*	5.8±0.6	12.3±3.3	6.9±0.3	7.9±1.2	8.6±2.5

Hct, hematocrit; [Hb], hemoglobin concentration (mmol l^{-1}); MCHC, mean corpuscular hemoglobin concentration (mmol l^{-1}).

Values represent means±s.e.m. Symbols denote a statistically significant difference from NCFW (*) or between NCSW and HCSW (‡) at the respective time point.

et al., 2003), and an increase in NKA activity during exposure to hypercarbia has been observed at the same level of hypercarbia used here in white sturgeon held in FW (Baker et al., 2009).

Possible interaction of osmoregulation and acid–base balance in net Na^+ and Cl^- transport

Plasma ‘strong ion difference’ ([strong cations]–[strong anions]) has been used to describe changes in acid–base status (Smatresk and Cameron, 1982; Tang et al., 1988; Maxime et al., 1991; Whiteley et al., 2001; reviewed by Truchot, 1987). In this study, we calculated the difference in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ (the predominant cation and anion in the blood plasma and in calculating the strong ion difference) as ‘ $\text{Na}–\text{Cl}$ ’. Plasma $\text{Na}–\text{Cl}$ values presented here were positive in all sturgeon throughout the 10 day experiment, which is reflective of the expected higher $[\text{Na}^+]$ than $[\text{Cl}^-]$ in the plasma compartment. Despite large fluctuations in both plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ during the first few days of SW acclimation, plasma $\text{Na}–\text{Cl}$ remained relatively stable in NCSW sturgeon. However, this ratio was not preserved in HCSW sturgeon, as there were two distinct and significant increases in plasma $\text{Na}–\text{Cl}$ (days 0–1 and 7–10). The timing of these changes corresponded to two distinct bouts of pH_e recovery, and throughout the experiment, plasma $\text{Na}–\text{Cl}$ was strongly correlated with plasma $[\text{HCO}_3^-]$ in HCSW (but not NCFW or NCSW).

The increase in $\text{Na}–\text{Cl}$ in the first 24 h after salinity exposure (i.e. the first bout of pH_e recovery) can be attributed to the HCSW sturgeon having accumulated relatively more Na^+ than Cl^- during this initial salt-loading period of the SW acclimation. The apparent association between pH_e recovery and a relative increase of Na^+ accumulation during this time may be the result of inward Na^+ transport being tied to outward H^+ transport via an apical NHE or an apical H^+ -ATPase with associated Na^+ channel (Claiborne and Heisler, 1984; Iwama and Heisler, 1991; Dymowska et al., 2014), or the co-transport of Na^+ and HCO_3^- into the extracellular compartment via a basolateral $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBC1) (Hirata et al., 2003; Perry et al., 2003).

Although a relative increase in Na^+ influx immediately following salinity exposure may have briefly aided in pH_e recovery, such inward transport of Na^+ is counter-productive to the outward direction of Na^+ transport required for SW acclimation. As the sturgeon then made the physiological adjustments necessary to acclimate to SW and recover plasma osmolality (presumably including a molecular reorganization of the gill epithelium in order to excrete Na^+ and Cl^-), this may have been to the detriment of any further pH_e recovery. This might

explain the plateau of pH_e recovery during days 1–7 after SW exposure, as well as the return of plasma $\text{Na}–\text{Cl}$ nearer to control values during this time. This stagnation of pH_e recovery may also imply a prioritization of osmoregulatory recovery over acid–base recovery in this period of combined exposure to acid–base and ionoregulatory disturbances.

The second significant increase in plasma $\text{Na}–\text{Cl}$ (and second bout of pH_e recovery) in HCSW (days 7–10) appeared to be the result of a significant reduction of plasma $[\text{Cl}^-]$ relative to $[\text{Na}^+]$. The removal of Cl^- from the extracellular compartment in exchange for HCO_3^- may be associated with a basolateral $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger in acid-secreting epithelial cell types (Perry and Gilmour, 2006). The common requirement and upregulation of gill epithelium phenotypes for SW acclimation and acidosis compensation leading to cooperative net plasma Cl^- removal might explain the dramatic reduction in plasma $[\text{Cl}^-]$ during this final bout of pH_e recovery (days 7–10) in HCSW.

Conclusions

This study describes the time course of SW acclimation by juvenile white sturgeon. The 2 year old age class in the present study represents the youngest age class of white sturgeon examined to date that has been able to make the physiological adjustments necessary to hypo-osmoregulate in elevated salinity. Exposure to elevated dissolved CO_2 significantly increases the osmotic disturbance incurred during acclimation to SW. Exposure to elevated dissolved CO_2 also affects the ionic composition of the blood plasma during SW acclimation, and these differences in ionic composition appear to be related to acid–base status. The associations of relative plasma Na^+ accumulation and relative plasma Cl^- removal with pH_e recovery substantiate similar findings from other investigations into the relationship between Na^+ and Cl^- transport and acid–base balance. Unique to this study are the description of a potential prioritization of osmoregulatory recovery over acid–base recovery during coincidental acid–base and ionoregulatory requirements, and the description of pH_e recovery associating more prominently with plasma Na^+ accumulation prior to osmotic recovery and more prominently with plasma Cl^- removal following osmotic recovery, as the requirements for osmoregulation transition from ion uptake to ion excretion throughout SW acclimation.

MATERIALS AND METHODS

Animal care and rearing conditions

All experiments were performed at an indoor facility at the International Centre for Sturgeon Studies (ICSS), Vancouver Island University (VIU),

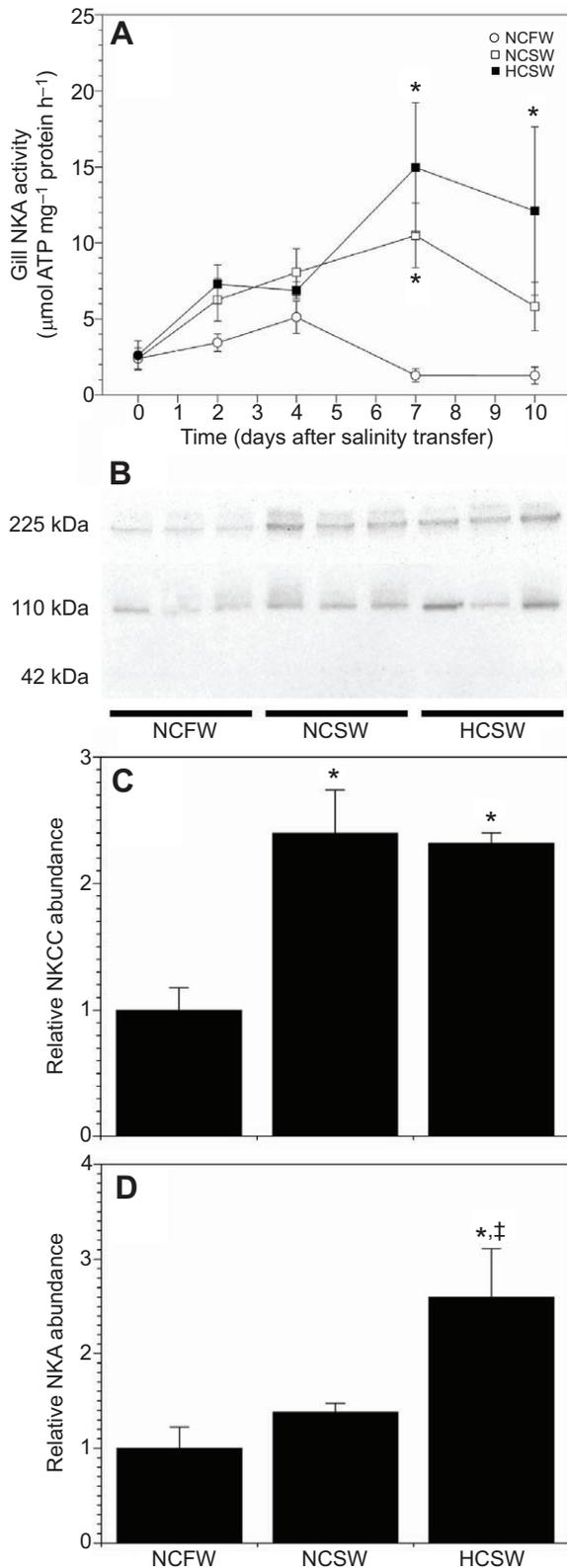


Fig. 3. Gill protein activity and abundance. (A) Gill Na^+/K^+ -ATPase (NKA) activity in white sturgeon acclimated to NCFW, NCSW or HCSW over 10 days. (B–D) Western blot (B), and relative gill protein abundance of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC; 225 kDa; C) and NKA α -subunit (110 kDa; D) against a β -actin (42 kDa) loading control on day 4 of SW acclimation. Values in A, C and D are presented as means \pm s.e.m. Symbols denote a statistically significant difference from NCFW (*) or between NCSW and HCSW (\ddagger) at the respective time point.

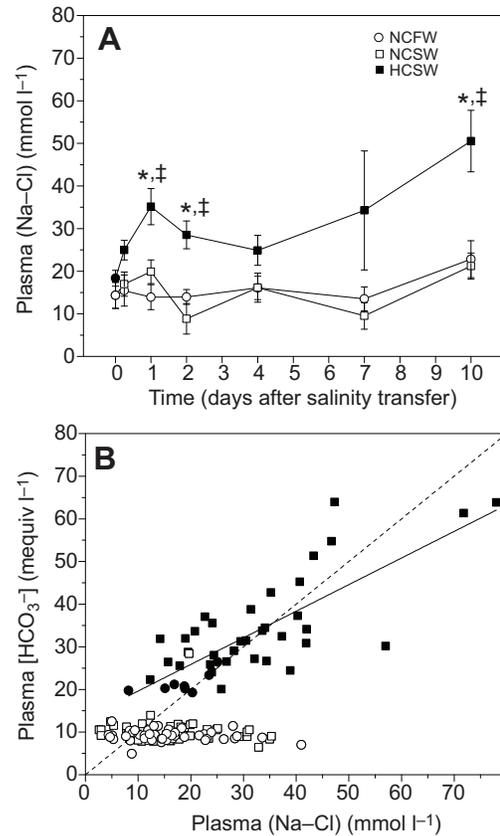


Fig. 4. Difference in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Na-Cl) and relationship between plasma Na-Cl and $[\text{HCO}_3^-]$ in white sturgeon acclimated to NCFW, NCSW or HCSW over 10 days. (A) Plasma Na-Cl. (B) Relationship between plasma Na-Cl and $[\text{HCO}_3^-]$. Values in A represent means \pm s.e.m. Symbols denote a statistically significant difference from NCFW (*) or between NCSW and HCSW (\ddagger) at the respective time point. HCSW correlation in B is described by the solid line ($m=0.63, r^2=0.77, P<0.001$), and the dashed line displays the line of identity ($m=1$).

Nanaimo, BC, Canada. Juvenile white sturgeon (2 years post-hatch; 38.1 ± 0.3 cm, 339.4 ± 7.2 g) were reared at the ICSS from eggs procured from a local white sturgeon aquaculture brood stock (Fraser River, BC, Canada origin). All sturgeon had been maintained in large indoor flow-through tanks in dechlorinated FW at $\sim 18^\circ\text{C}$ under a simulated natural photoperiod for at least 6 months prior to experimentation, and were fed to satiation daily. The white sturgeon used here were not exposed to elevated salinity or P_{wCO_2} prior to this study. All procedures were performed according to Animal Usage Protocols vetted and accepted by the Canadian Council on Animal Care (VIU) and Institutional Animal Care and Use Committee (DePaul University).

Experimental procedure

Food was withheld for 48 h and sturgeon were removed from their rearing tanks and randomly assigned (54 fish per tank) to one of three large (2000 l) circular, darkened fiberglass tanks with recirculating filtration systems maintained at $18.5 \pm 0.2^\circ\text{C}$ and well oxygenated ($P_{\text{wO}_2} > 15.0$ kPa). Prior to 'day 0' of SW acclimation, sturgeon were exposed to either normocarbic (ambient $P_{\text{wCO}_2} < 0.03$ kPa) or hypercarbic (elevated $P_{\text{wCO}_2} \approx 6$ kPa) FW for 24 h. A P_{wCO_2} tension of 6 kPa was chosen to be consistent with previous hyperbaria experimentation on white sturgeon (Baker et al., 2009), and a salinity of 20‰ [prepared by mixing dechlorinated municipal FW with filtered (32 μm) local marine water ($\sim 30\%$; referred to as 'SW')] was chosen to minimize osmotic stress but to ensure the requirement to hypo-osmoregulate (the latter confirmed by preliminary experimentation). Implementation of hypercarbic conditions prior to SW exposure (versus a

simultaneous exposure) was preferred in order to minimize the overall stress on the fish. Thus, experimentation consisted of a 10 day exposure to the following treatments: (1) normocarbic freshwater (NCFW); (2) normocarbic seawater (NCSW); and (3) hypercarbic seawater (HCSW). In all treatments, feeding was withheld to eliminate this as a potentially confounding variable in the study.

The experimental P_{WCO_2} of 6 kPa was achieved using a P_{CO_2} /pH feedback controller (DAQ-M, Loligo Systems Inc., Tjele, Denmark) connected to a pH meter (WTW pH 3310, Loligo Systems Inc.) and pH electrode (SenTix 41, Loligo Systems Inc.), and controlled using CapCTRL software (Loligo Systems Inc.). When P_{WCO_2} of 6 kPa (as inferred from pH, based upon a previously determined relationship between pH and P_{WCO_2} in white sturgeon; Baker et al., 2009) dropped below the target value (as indicated by pH increasing above a target value), the system would bubble pure CO_2 gas via an air stone into the tank until P_{WCO_2} was returned to the desired level.

Sampling protocol

At 6 h and 1, 2, 4, 7 and 10 days following SW transfer (or in the FW control), eight sturgeon from each of the three control or experimental conditions were individually killed with MS-222 (~200 mg l⁻¹; buffered with NaHCO₃) and terminally sampled. Mass and fork length were measured, and a 3 ml aliquot of blood was collected via caudal puncture using a chilled heparinized needle (22 G) and syringe (3 ml). Blood was divided into two aliquots. The first was used to measure blood pH (considered pH_c), and the second was used for Hct and [Hb]. The remaining blood was centrifuged (5 min at 3000 g) and the plasma was collected and stored for measurement of total CO₂ (TCO₂), plasma osmolality and individual plasma ion (Na⁺ and Cl⁻) concentrations.

Following blood sampling, gill and white muscle samples were rapidly excised, blotted dry, flash frozen in liquid nitrogen and then stored at -80°C for later analysis. A second white muscle sample was blotted dry, weighed (considered 'wet mass') and placed in a drying oven (60°C) for determination of white muscle water content.

Blood analyses

Whole blood pH was measured immediately after sampling (Thermo Scientific Orion Benchtop pH meter, Fisher Scientific Inc., Waltham, MA, USA). [Hb] was determined spectrophotometrically following dilution in Drabkin's reagent (following the manufacturer's instructions; Sigma-Aldrich, St Louis, MO, USA). Plasma TCO₂ was determined using a CO₂ analyzer (Model 965 Analyzer, Ciba-Corning Canada Inc., Markham, ON, Canada). Plasma osmolality was determined using a vapor pressure osmometer (VAPRO 5600, Wescor, Inc., Logan, UT, USA), plasma [Na⁺] was measured with a flame photometer (Jenway PFP7, Bibby Scientific Ltd, Staffordshire, UK) and plasma [Cl⁻] was measured with a ChloroChek chloridometer (Wescor, Inc., Logan, UT, USA).

Gill NKA activity

Gill filaments were homogenized on ice in SEID buffer (150 mmol l⁻¹ sucrose, 10 mmol l⁻¹ EDTA, 50 mmol l⁻¹ imidazole and 0.1% sodium deoxycholate, pH 7.5) by hand using a ground glass homogenizer, and centrifuged for 2 min (4000 g, 4°C) to remove filaments and other insoluble material. The supernatant was used directly in the assay of enzyme activity.

NKA activity was determined spectrophotometrically using an NADH-linked assay as described in Bystriansky et al. (2006), modified from the methods of Gibbs and Somero (1990) and McCormick (1993). Briefly, ADP formed from the hydrolysis of ATP by NKA was enzymatically coupled to the oxidation of reduced NADH using commercial preparations of pyruvate kinase and lactate dehydrogenase. Gill samples were assayed for ATPase activity in the presence and absence of the NKA-specific inhibitor ouabain (1 mmol l⁻¹). The difference in the rate of NADH oxidation ($\lambda=340$ nm) between the inhibited solution B and uninhibited control solution A was used to calculate NKA-specific activity. The reaction was performed at 25°C and analyzed using a microplate spectrophotometer (SPECTRAMax PLUS 384, Molecular Devices Corp., Sunnyvale, CA, USA). NKA activity was expressed as $\mu\text{mol ATP h}^{-1} \text{mg}^{-1}$ protein, where the protein content of the

crude homogenates was determined spectrophotometrically ($\lambda=595$ nm) as described by Bradford (1976) using a bovine serum albumin standard. All reagents listed were purchased from Sigma-Aldrich.

Immunoblotting

Gill tissue homogenates were prepared and protein content measured similar to the methods described above, differing only slightly in the homogenization buffer: 25 mmol l⁻¹ Tris-HCl (pH 7.6), 150 mmol l⁻¹ NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and 5 $\mu\text{mol l}^{-1}$ protease inhibitor cocktail (Sigma-Aldrich). Protein samples (10 μg) were then made up as a 1:1 solution with Laemmli loading buffer and electrophoretically separated in precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA), and transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were washed in PBST, blocked (in 5% non-fat milk in PBST) then incubated overnight at 4°C in a 1:1000 dilution of the primary antibody: mouse monoclonal anti- β -actin (as a loading control), mouse monoclonal anti-NKA α -subunit ($\alpha 5$) or mouse monoclonal anti-NKCC1 (T4) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The use of the T4 antibody for NKCC1 detection in the white sturgeon gill is novel. Although the T4 antibody is known to also recognize the Na⁺/Cl⁻ co-transporter (NCC), which is almost exclusively expressed in FW (not SW) gill phenotypes (Hiroi et al., 2008), the T4 antibody has been widely used in analysis of gill NKCC abundance (Evans et al., 2005), including analysis of gill NKCC abundance in green sturgeon (*Acipenser medirostris*) (Sardella and Kültz, 2009). After primary antibody incubation, membranes were washed with PBST then incubated for 1 h at 23°C in a 1:5000 dilution of a secondary antibody (goat anti-mouse). Labeling was detected by enhanced chemiluminescence (Alpha Innotech Fluor Chem HD2). Relative NKA and NKCC band intensity was analyzed using ImageJ 1.48 (National Institutes of Health, Bethesda, MD, USA).

Calculations and statistical analyses

White muscle samples were dried to a stable mass (considered 'dry mass') and percentage water content was calculated as: [(wet mass-dry mass)×100]/wet mass. MCHC was calculated as: ([Hb]÷Hct)×100. P_{aCO_2} and plasma [HCO₃⁻] were calculated from TCO₂ and pH_c measurements as described by Brauner et al. (2004), using the CO₂ solubility coefficient (α_{CO_2}) and pK' for rainbow trout (Boutilier et al., 1984) and a reorganization of the Henderson-Hasselbalch equation. Differential changes in plasma Na⁺ and Cl⁻ (Na-Cl) were calculated as [Na⁺]-[Cl⁻] as a means of describing differential changes in ion concentrations. Plasma Na-Cl was regressed with plasma [HCO₃⁻] and a line of best fit was calculated as a means of comparing ionoregulatory status with acid-base status.

All values are presented as means±s.e.m. (N=6–8), except for HCSW on day 10, where N=4. A two-way ANOVA analysis was used for comparison between treatments across time, and a Holm-Šidák *post hoc* analysis was used to identify significant differences between treatments at each time point. An α -value of 0.05 was selected to denote statistical significance in all analyses. All statistical tests were performed using Stata 12.0 (StataCorp LP, College Station, TX, USA); all figures were assembled using OriginPro 9.0 (OriginLab Corp., Northampton, MA, USA).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.A.S. and J.S.B. designed the experiment. C.A.S., D.W.B. and J.S.B. executed the experiment. C.A.S., D.W.B. and J.S.B. analyzed the data. All authors interpreted the results and provided editorial feedback. C.A.S. and J.S.B. wrote the article.

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