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PHYSIOLOGICAL EFFECTS OF AQUATIC HYPERCARBIA ON SEAWATER ACCLIMATION IN THE WHITE STURGEON (ACIPENSER TRANSMONTANUS)

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PHYSIOLOGICAL EFFECTS OF AQUATIC HYPERCARBIA ON SEAWATER ACCLIMATION IN THE WHITE STURGEON (ACIPENSER TRANSMONTANUS)

A Thesis Presented in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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by

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PREFACE

Chapter 1 General Introduction

Comments: This chapter was written by Ciaran A. S. Shaughnessy under the supervision and editorial advisement of Dr. Jason S. Bystriansky.

Chapter 2 Interaction of osmoregulatory and acid-base compensation in white sturgeon (Acipenser transmontanus) during exposure to aquatic hypercarbia and elevated salinity

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Ethical Note

All live animal usage protocols were vetted and accepted by Institutional Animal Care and Use Committee at DePaul University and the Canadian Council on Animal Care at Vancouver Island University.
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ABSTRACT

Migratory fishes encounter a variety of environmental conditions throughout their life, including changes in salinity, temperature, and dissolved gases. It is important to understand how these fishes are able to acclimate to simultaneous environmental stressors. This thesis 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*). Many ion transport mechanisms at the gill involved with acid-base compensation are also required for the regulation of plasma Na$^+$ and Cl$^-$, the predominant extracellular ions. Thus, the interaction between iono- and acid-base regulation has been hypothesized. In this thesis, blood pH (pHe), plasma ion concentrations, white muscle water content, and gill Na$^+$/K$^+$-ATPase (NKA) and Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) abundance were examined over a 10-day seawater (SW) acclimation period under normocarbia (NCSW) or during prior and continued exposure to hypercarbia (HCSW), and compared to a normocarbic 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 osmolarity over NCSW by day 2 of SW exposure. Interestingly, pHe 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.
1 General introduction

1.1 Introduction

Throughout their lives, migratory fishes experience a wide range of biotic and abiotic conditions that illicit behavioral and physiological responses. These include changes in environmental conditions associated with their migration (which fishes might encounter relatively abruptly, such as changes in salinity, PCO$_2$, PO$_2$, temperature, and pH), more gradual environmental changes over seasons (such as temperature and photoperiod), hormonal and developmental changes (which they may experience in preparation for and during migration), and changes in predation pressure and food availability. Together, tolerance, acclimation, and adaptation to these changes define survival as well as the fundamental and realized niches for these animals. Additionally, because fishes in the wild are likely to experience more than one of these changing conditions at any given time, it is important to understand how simultaneous environmental stressors might interact on the physiology of these animals. In the introductory chapter of this thesis, the physiological responses by fishes to changes in salinity and PCO$_2$ are reviewed, the importance of investigating the physiological interaction of multiple environmental stressors is discussed, and the white sturgeon (*Acipenser transmontanus*) is introduced as a valuable study species in which to explore the interaction of salinity and PCO$_2$ stressors.
1.2 Osmoregulation, anadromy, and seawater acclimation in fishes

Most fish inhabit freshwater or marine habitats. Freshwater environments (e.g., lakes and rivers) have very low salinity (~0.01 ‰; 25 mOsm L⁻¹) and contain the major ions: HCO₃⁻ (~1.7 mM), Ca²⁺ (~0.8 mM), Na⁺ (~0.05 mM), and Cl⁻ (~0.05 mM). Marine environments, on the other hand, have much higher salinity (~35 ‰; 1025 mOsm L⁻¹) and are heavily concentrated with Na⁺ (~470 mM) and Cl⁻ (~548 mM) ions. Where freshwater meets the sea (i.e., estuaries, salt marshes), the environmental salinity falls along a spectrum between ‘freshwater’ and ‘marine’ (termed ‘brackish’) depending on the location in the estuary and the tidal cycle.

The environmental salinity in which an aquatic organism lives can present significant ionic and osmotic challenges. For example, cell volume is highly dependent on the concentration of solutes (including Na⁺ and Cl⁻) in the extracellular compartment (i.e., blood). If the concentration of extracellular solutes suddenly changes, water will enter or exit the cell via osmosis leading to cell swelling or shrinkage, thus damaging the cell and interrupting normal cellular function. Aquatic animals have evolved various strategies in coping with these osmotic challenges. For example, many marine invertebrates (as well as hagfish and elasmobranchs) are ‘osmoconformers’ in that they have evolved to maintain an osmolality of extracellular fluids similar to that of their environment. Although the strategy of osmoconforming is potentially problematic if the environmental salinity were to suddenly change, it is adaptive due to the highly stable nature of the marine environment. On the other hand, most fishes (with the exception of hagfish and elasmobranchs) are ‘osmoregulators’ in that they regulate their internal osmotic composition different from that of their environment, maintaining an internal
osmolality of approximately one-third that of seawater. This means that in a marine environment, fishes are challenged by the osmotic loss of water to (and passive gain of ions from) the more solute-concentrated marine environment; the opposite is true for fishes in freshwater, which are challenged by the osmotic gain of water from (and passive loss of ions to) the more dilute environment.

Of the osmoregulating fishes, most species are considered ‘stenohaline’ in that they cannot tolerate large fluctuations in environmental salinity. These fishes only have the capacity to either ‘hypoosmoregulate’ (regulate internal ion concentrations below that of their environment; *i.e.*, marine fishes) or ‘hyperosmoregulate’ (regulate internal ion concentrations above that of their environment; *i.e.*, freshwater fishes), but not both. As a result, these fishes live in relatively stable salinity conditions and do not migrate outside of these habitats throughout their lifetime (*i.e.*, exclusively freshwater or exclusively marine). On the other hand, some fishes are ‘euryhaline’, capable of tolerating large changes in environmental salinity. Examples of euryhaline lifestyles include inhabiting waters which experience large fluctuations in salinity (such as estuaries) and/or migrating between freshwater and marine habitats (such as by anadromous fishes).

Anadromous fishes—which include members of the families: Salmonidae (salmon, trout), Petromyzontidae (lamprey), Clupeidae (herring), and Acipenseridae (sturgeon)—have a relatively unique life history. These fishes are born in freshwater habitats, then, as juveniles, migrate into resource abundant brackish or marine waters to develop into adulthood. Once they reach sexual maturity and are ready to spawn, anadromous fishes migrate back upstream to spawn in the freshwater habitats of their origin. Although migrating between freshwater and marine habitats may confer some
ecological advantages (i.e., lower threat of predation as juveniles in freshwater habitats and greater food abundance for adults in marine habitats), it requires the physiologically challenging process of switching between hyperosmoregulating (uptaking ions) in freshwater and hypoosmoregulating (excreting ions) in seawater. The maintenance of relatively high internal ion concentrations in dilute freshwater or relatively low internal ion concentrations in concentrated seawater is achieved through a combination of organismal and cellular processes.

In freshwater, fishes passively gain water and lose ions. To counter this, they have low drinking rates (Bath and Eddy, 1979), and high glomerular filtration rates, and produce large amounts of dilute urine (Jobling, 1995), while actively absorbing ions (primarily Na\(^+\) and Cl\(^-\)) across the gill epithelium via a suite of ion transport proteins. The transport of Na\(^+\) and Cl\(^-\) against a steep concentration gradient between internal and external ion concentrations is facilitated by the creation of favorable local electrogenic gradients across the gill epithelium. It is currently accepted that uptake of Na\(^+\) and Cl\(^-\) is associated with the excretion of H\(^+\) and HCO\(_3^\)\(^-\), which is facilitated by the local electrogenic gradients produced by the activities of the apically-located vacuolar-type H\(^+\)-ATPase (VHA) and the basolaterally-located Na\(^+\)/K\(^+\)-ATPase (NKA) (Avella and Bornancin, 1989).

In this process (Fig. 1.1), inward Na\(^+\) transport is tied to outward H\(^+\) transport via an apical Na\(^+\)/H\(^+\) exchanger (NHE) or a Na\(^+\) channel associated with the VHA (Claiborne and Heisler, 1984; Iwama and Heisler, 1991; Dymowska et al., 2014). It is thought that the NHE and VHA/Na\(^+\) channel complex are not typically expressed together; fishes either utilize one mode of Na\(^+\)/H\(^+\) exchange or the other, but not both (Evans et al., 2005).
Na\(^+\) is transported into the blood stream via the NKA, which in doing so, actively maintains a low intracellular Na\(^+\) concentration (which also aids in the passive Na\(^+\) uptake via the NHE or VHA-associated Na\(^+\) channel) and high intracellular K\(^+\) concentration. To achieve this, the NKA transports 3 Na\(^+\) from inside of the cell out into the blood in exchange for 2 K\(^+\), which recycle from the cell back into the blood through a basolateral K\(^+\) channel. Additionally, Cl\(^-\) ions are absorbed from freshwater into the cell via an apically-located Cl\(^-\)/ HCO\(_3^-\) exchanger (AE). Although the basolateral transport of Cl\(^-\) into the blood has yet to be fully described, it is expected that Cl\(^-\) passively moves into the blood via a basolateral Cl\(^-\) channel (Evans et al., 2005).

In seawater, fish passively lose water while gaining ions. To counter this, they increase their drinking rate, decrease their glomerular filtration rate in order to release large amounts of isomotic urine, and actively secrete ions (again, primarily Na\(^+\) and Cl\(^-\)) across the gill epithelium. Similar to ion uptake in freshwater, ion excretion in seawater is facilitated by the electrogenic gradients produced by the NKA; a large inward gradient for Na\(^+\) produced by the NKA is exploited for Cl\(^-\) excretion (Silva, 1977) (Fig. 1.2). A basolateral Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter (NKCC) links the passive inward transport of Na\(^+\) (down a concentration gradient) with the inward transport of K\(^+\) (equimolar) and Cl\(^-\) (bimolar) (up a concentration gradient). Through NKCC transport, intracellular Cl\(^-\) concentration becomes great enough that Cl\(^-\) can passively exit across an apically-located Cl\(^-\) channel (CFTR: cystic fibrosis transmembrane regulator). The outward movement of Cl\(^-\) produces an outside-negative/extracellular-positive electrogenic gradient which drives locally-accumulated plasma Na\(^+\) (due to local NKA activity) to exit the blood via a paracellular pathway (‘leaky junction’).
1.3 Aquatic hypercarbia and acid-base regulation in fishes

Typical ambient (‘normal’) partial pressure of CO$_2$ in water (PwCO$_2$) is approximately 0.02 kPa PCO$_2$. However, PwCO$_2$ can vary substantially between aquatic systems, depending on a range of abiotic and biotic factors. Aquatic hypercarbia (elevated PwCO$_2$) can occur in marine, estuarine, and fresh waters as a result of both natural and anthropogenic stimuli. Naturally-occurring aquatic hypercarbia can be attributed to a number of factors. In marine environments, aquatic hypercarbia (on the order of 0.5 – 1.5 kPa) occurs regularly in tide pools (Burggren and Roberts, 1991), at depths of 200–500 m (Heisler, 1986), and as a result of carbon sequestration (Seibel and Walsh, 2003). In freshwater and brackish environments, aquatic hypercarbia can be much more severe (up to 8 kPa; Heisler, 1982; Ultsch, 1996; Burnett, 1997). In these each of these environments, nighttime oxygen consumption and CO$_2$ production by aquatic flora can lead to decreases in water pH by over 1 pH unit (Burnett, 1997), which can be exacerbated by poor water mixing (such as in slow-moving rivers or river eddies, seasonal ponds, tide pools, etc.).

With the rise of land-based aquaculture and industrial CO$_2$ emissions in the last half-century, there is a growing interest to investigate potential anthropogenic causes for aquatic hypercarbia, and its effects on aquatic organisms. Land-based, recirculating aquaculture systems can expose fishes to higher-than-natural levels of aquatic hypercarbia. Often, oxygen is artificially supplied to these systems to increase biomass production; however, an increase in biomass leads to an increase in CO$_2$ production, and without a specific method for CO$_2$ removal, this can result in severe aquatic hypercarbia. Likewise, industrial emission of CO$_2$ is also expected to lead to aquatic hypercarbia.
Marine surface waters are responsible for absorbing one-third to one-half of the annual emission of CO₂ by human activity (Intergovernmental Panel on Climate Change (IPCC), 2007). With the current estimations of atmospheric CO₂ concentration exceeding 380 ppm (which is likely higher than at any point in the last 10 million years; IPCC, 2007), it is expected that surface-water PwCO₂ levels will rise nearly five-fold, decreasing marine surface water pH by 0.7 units (Caldeira and Wickett, 2003; IPCC, 2007), a phenomenon commonly referred to as ‘ocean acidification’.

In addition to its ecological relevance as a natural and anthropogenic environmental stressor, aquatic hypercarbia also represents an historical laboratory challenge utilized by physiologists to study mechanisms of acid-base regulation. Regulation of intracellular and extracellular pH (pHi and pHe, respectively) is crucial to survival in most vertebrate species (Boron and De Weer, 1976; Heisler, 1986). Loss of pHi homeostasis in either direction (basic or acidic) can alter local charges on proteins and diminish protein function, disrupting many important cellular processes including signaling, volume regulation (i.e., osmoregulation), and protein expression (Putnam and Roos, 1997). Although most cells have some capacity to regulate pHi, the primary strategy utilized by most water-breathing ectotherms (i.e., fishes and aquatic invertebrates) for maintaining pHi homeostasis is active regulation of pHe (i.e., blood pH), which reduces the pressure on individual cells to regulate pHi (Truchot, 1987).

Vertebrates compensate for acute extracellular acid-base disturbances in a number of ways: (1) by immediate physiochemical buffering with bicarbonate and non-bicarbonate buffers, (2) by active respiratory (i.e., ventilatory) adjustments to affect arterial PCO₂ (PaCO₂) via the CO₂–HCO₃⁻ buffer system, or (3) by active net transport of
acids-base equivalents between the cell and extracellular fluids, and/or extracellular fluids and the environment (Evans et al., 2005). The obligatory high ventilation rates in fishes, results in a relatively low physiological PaCO₂ (0.15 kPa in normocarbic water) and arterial [HCO₃⁻] compared to air-breathing vertebrates (PaCO₂ > 5 kPa in mammals) (Truchot, 1987). Therefore, fishes exclusively using an aqueous medium for respiration (i.e., not air-breathing fishes) have a generally low buffer capacity of the extracellular compartment, and adjustments in ventilation can only minimally affect PaCO₂. So, in exclusive water-breathers, the effectiveness of the above-mentioned mechanisms (1) and (2) to regulate acid-base status is highly limited, thus increasing the importance of mechanism (3), the net exchange of acid-base equivalents with the environment, in these fishes.

The onset and recovery of an acidosis in fishes during aquatic hypercarbia are explained by the hydration of CO₂ in water to form carbonic acid (H₂CO₃) (Eq. 1), as well as an application of the Henderson-Hasselbalch equation (Eq. 2):

\[
\text{blood } pH = pK_{a\text{carbonic acid}} + \log \left( \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \right) \quad \text{Equation 2}
\]

Gaseous CO₂ dissolved in the respiratory medium (i.e., water) reacts with H₂O to form H₂CO₃, which then dissociates to produce a 1:1 ratio of liberated HCO₃⁻ and H⁺ (Eq. 1). Physiologically, the production of a 1:1 ratio of HCO₃⁻ to H⁺ actually results in a net reduction of blood pH (explained by Eq. 2; where \( pK_{a\text{carbonic acid}} \)) is the acid
dissociation constant of carbonic acid). For example, assuming a ‘normal’ homeostatic blood pH in fishes to be pH 7.8 (although this varies with temperature) and given that \( pK_a(\text{carbonic acid}) \) is 6.1, then, following Eq. 2, \( \log \left( \frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} \right) = 1.7 \). This implies that fish must maintain the ratio of extracellular \([\text{HCO}_3^-] \) to \([\text{H}^+] \) at approximately 50:1 to maintain a homeostatic blood pH of 7.8. This is relatively high compared to mammals \(( [\text{HCO}_3^-]:[\text{H}^+] \) in mammals is approximately 20:1), in which homeostatic blood pH is closer to 7.4. Thus, the passive accumulation of a 1:1 ratio of \( \text{HCO}_3^- \) to \( \text{H}^+ \) (such as during aquatic hypercarbia) will decrease this homeostatic ratio, thus increasing the overall \([\text{H}^+] \) and depressing blood pH.

Alternatively, as \([\text{HCO}_3^-] \) is increased, it acts as a buffer in the system, shifting the equilibrium in Eq. 1 back to the left, reducing liberated \( \text{H}^+ \), thus recovering blood pH. This hydration of \( \text{CO}_2 \) and dehydration of carbonic acid are generally slow chemical reactions, but intracellular carbonic anhydrase (CA) can act as catalyst to increase the rate of these reactions.

In response to aquatic hypercarbia, pH regulation in water-breathing fishes is generally divided into two parts—an initial respiratory acidosis followed by a pH recovery over days. The initial acidosis is the result of \( \text{PaCO}_2 \) immediately equilibrating with \( \text{PwCO}_2 \), thus decreasing blood pH (pHe) (as described by Eq. 1). Following acidosis, pHe recovery is achieved by transport of acid-base molecular equivalents across the gill epithelium (Brauner and Baker, 2009)—acid equivalents (primarily \( \text{H}^+ \)) are excreted out of the body and base equivalents (primarily \( \text{HCO}_3^- \)) are accumulated in the extracellular compartment. In fishes, much like in osmoregulation, these ion transport processes are carried out primarily across the gill epithelium.
As CO₂ dissolved in the blood diffuses across the basolateral membrane of gill epithelial cells, it is converted by carbonic anhydrase to a 1:1 ratio of H⁺ and HCO₃⁻, thus leading to a potential extracellular acidosis. To compensate for this acidosis, H⁺ is excreted out of the body across the apical membrane, and HCO₃⁻ is accumulated in the blood via transport across the basolateral membrane of the gill epithelium (Fig. 1.3). Branchial H⁺ excretion across the apical membrane occurs by two possible mechanisms: either H⁺ is actively excreted via a VHA (which is usually associated with an equimolar uptake of Na⁺ via an adjacent Na⁺ channel); or an equimolar exchange of H⁺ (outward) and Na⁺ (inward) occurs via an apical NHE, (Claiborne and Heisler, 1984; Iwama and Heisler, 1991). It is important to note that both of these mechanisms of H⁺ excretion are tied to Na⁺ uptake, which is aided by the low intracellular Na⁺ concentration produced by the activity of the NKA. The extracellular accumulation of HCO₃⁻ is achieved by one of two pathways across the basolateral membrane: either HCO₃⁻ is transported in an equimolar exchange for plasma Cl⁻ via a basolateral AE (Cl⁻ is then recycled back into the extracellular space via a basolateral Cl⁻ channel); or HCO₃⁻ is transported into the extracellular compartment alongside Na⁺ via a basolateral Na⁺/HCO₃⁻ co-transporter (NBC) (Hirata et al., 2003; Perry et al., 2003).

1.4 The importance of physiological analysis of multiple stressors

Fishes inhabit a wide range of environments. Migratory fishes and estuarine fishes are unique in that they experience large changes to their environment throughout their life. The behavioral and physiological responses by fishes to isolated changes in the biotic and abiotic factors mentioned above are relatively well-studied. That is, there have
been a number of studies addressing salinity acclimation, acid-base regulation, thermoregulation, developmental changes, ecological pressures, etc. in fishes. However, at any given time, the behavior and/or physiology of fishes in the wild will likely be challenged by two or more of these factors acting simultaneously (e.g., hypoxic waters are usually also more acidic, Burnett, 1997; thermal tolerance is likely controlled by a limitation in oxygen supply, Pörtner and Knust, 2007; or, intuitively, migrating between different abiotic conditions is likely associated with changes in ecological condition).

In this light, Todgham and Stillman (2013) describe the necessity for organismal biologists to examine the effects of multiple environmental stressors, particularly for those seeking to make ecological inferences about the organismal effects of environmental factors. That is, the physiological responses to each of the many simultaneous stressors an organism experiences at any given time likely interact, and identifying the nature of these physiological interactions is imperative in understanding the true biology of that organism in the wild. This is certainly the case in evaluating the physiological experience of a migrating fish.

The multitude of simultaneous changes in the abiotic environment during a downstream migration made by an anadromous fish is a salient example of the need for more multiple stress analyses in physiological research. Estuarine and migrating organisms are continuously experiencing an array of environmental challenges, and because multiple stressor analysis attempts to offer a more comprehensive view of the physiology associated with these challenges, it may also bring to light physiological phenomena which would have otherwise been unobservable by studying single environmental stressors in isolation. For example, during seawater entry, anadromous
fish may be more sensitive to changes in temperature, PO\textsubscript{2}, or PwCO\textsubscript{2} than they would be otherwise, but this can only be known via a multiple stress analysis of these environmental factors. Conversely, in colder times of year, estuarine fish may be slower to acclimate to changes in salinity, which can only be known by studying changes in temperature alongside changes in salinity in a multiple stressor experimental design.

This reasoning can be applied to the content of this thesis. For instance, it is clear that transport of the acid-base equivalents H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} during acid-base regulation is associated with transport of the osmotically active ions Na\textsuperscript{+} and Cl\textsuperscript{-}, the regulation of which are also important in osmoregulation (Fig. 1.4). Due to this potential association of osmoregulation and acid-base balance on ion transport, as well as a shared reliance on NKA activity, it is thought that these two physiological processes might interact in fishes under environmental pressure to simultaneously regulate both salinity-relevant ions and acid-base molecular equivalents. However, empirical evidence for the physiological interaction between acid-base balance and osmoregulation is still lacking.

It has been suggested that increased environmental salinity might be helpful during pHe recovery. Iwama and Heisler (1991) examined the effects of increased environmental [Na\textsuperscript{+}] on pHe recovery and found a minor increase in the rate of net H\textsuperscript{+} excretion via the NHE. However, research in this area has not yet examined the effects of exposure to aquatic hypercarbia on salinity acclimation. Indeed, the inward transport of Na\textsuperscript{+} across the gill during acidosis compensation might be counterproductive to the outward paracellular movement of Na\textsuperscript{+} required for successful seawater acclimation. By studying how osmoregulatory status in a fish changes during seawater acclimation under aquatic hypercarbia, and comparing these changes to those in fishes without a aquatic
hypercarbia challenge, the effect of aquatic hypercarbia on seawater acclimation can be described.

### 1.5 On the species, the white sturgeon (*Acipenser transmontanus*)

Sturgeon are among the largest and most primitive bony fish worldwide; the white sturgeon (*Acipenser transmontanus*) is the largest freshwater fish in North America and the third largest member of the sturgeon family Acipenseridae. Acipenseriform fishes are estimated to have radiated between 200 and 350 million years ago (Løvtrup, 1977; Nelson, 2006), making them among the most ancient of the actinopterygian fishes. Despite a relatively long history, morphological changes among the family have been minor (Krieger et al., 2008). This is most likely the result of a combination of long reproductive cycles (can be decades between spawning; Boreman, 1997), reduced predation due to their size (mature adults grow to 2-4 m in length) and unique body armoring (Auer, 2005), and low competition for benthic food resources (Miller, 2005). Since the proliferation of hydroelectric dams and the overfishing by caviar suppliers in recent decades, populations of sturgeon have been on a rapid decline. The International Union for Conservation of Nature (IUCN) has labeled over 85% of sturgeon populations worldwide as ‘at risk of extinction’. Although there has been an increase in conservation efforts surrounding the sturgeon family, still relatively little is known about the basic biology (*i.e.*, life cycle and physiology) of many sturgeon species, including the white sturgeon.

Most acipenserids are at least partially anadromous, living out their adult life in resource-abundant brackish or marine waters and returning upstream to spawn in
freshwater. However, some species of sturgeon have evolved exclusively freshwater existences, such as the lake sturgeon (*Acipenser fulvescens*), or have been forced to remain in freshwater by anthropogenic influence (*i.e.*, by the construction of hydroelectric dams), such as the case with the upper Columbia River white sturgeon subpopulation. Because white sturgeon can live out their life without ever making a seaward migration, and because the degree of their anadromy varies between subpopulations, white sturgeon are considered ‘semi-anadromous’.

Among the anadromous populations of white sturgeon, adults and older juveniles demonstrate a euryhaline lifestyle by inhabiting environments across the salinity spectrum (*i.e.*, freshwater, estuarine, and marine environments). During spawning, a female will deposit her eggs in a clean, moderate-velocity sections of a river to be fertilized by males and eventually hatch sheltered in the gravel of the river bottom (Bemis and Kynard, 1997). Planktonic larvae develop into free-swimming juveniles in these upstream, freshwater habitats. Juvenile white sturgeon spend many years in freshwater nursery habitats, and unlike more strongly-anadromous or obligatory-anadromous species, not every individual white sturgeon will make a seawater migration (Bemis and Kynard, 1997). Of those that do make a seawater migration, little is known about the timing (*i.e.*, age, developmental stage) of when white juveniles are capable of acclimating to the large change in salinity experienced when entering brackish waters. Evidence from ecological and physiological studies indicate juveniles smaller than 10-15 cm (less than 2 years old) are both absent from capture in brackish waters (North et al., 1993) and unable to acclimate to even dilute seawater (Amiri et al., 2009). Thus, the question of at what age juvenile white sturgeon are able to acclimate to brackish waters is still unresolved.
The white sturgeon is a particularly interesting species in which to address the questions explored in this thesis because in addition to being one of the most basal extant rayfinned fishes and an anadromous fish species, the white sturgeon is also tolerant to acid-base disturbances (Baker et al., 2009). It has been shown that white sturgeon are tolerant to severe aquatic hypercarbia (up to at least 6 kPa PwCO$_2$) (Baker et al., 2009). At lower PwCO$_2$ tensions (1.5 kPa PwCO$_2$), white sturgeon can effectively regulate pHe, but at high (6 kPa) PwCO$_2$ tensions, they have an uncommon ability (only known to exist in a small number of species, Brauner and Baker, 2009) to regulate pH$_i$ in most tissues while tolerating a large depression in blood pH (pHe reduced by ~0.7 pH units for at least 24 hours) (Baker et al., 2009).

1.6 Thesis objectives

Although particular mechanisms vary among fishes, it is clear that ion regulation involves the movement of acid-base molecular equivalents, and likewise, acid-base balance is reliant on the movement of Na$^+$ and Cl$^-$ ions. As such, the primary objective of this thesis was to investigate the physiological interaction between the processes of net ion secretion and acidosis compensation in a fish under simultaneous environmental salinity and hypercarbic challenges (i.e., during seawater acclimation under aquatic hypercarbia). The physiological interaction of osmoregulation and acid-base balance in this multiple stressor scenario is explored by examining whole-body and tissue-specific responses to these individual and combined environmental challenges.

This thesis addresses the questions: (1) Can two-year old white sturgeon acclimate to elevated salinity?; (2) Is seawater acclimation affected by aquatic hypercarbia?; and (3)
How do acidosis compensation and osmoregulatory compensation interact on the transport of Na$^+$ and Cl$^-$? Investigating these organismal and gill function adjustments to this multiple stressor scenario in a basal anadromous fish species will provide valuable information about the relationship between iono- and acid-base regulatory strategies, as well as provide an interesting evolutionary context for later comparisons with the more derived teleosts.
1.7 Figures

Figure 1.1: Schematic diagram of mechanisms for net uptake of Na\(^+\) and Cl\(^-\) ions from freshwater into the blood across the fish gill epithelium. AE: Cl\(^-\)/HCO\(_3\)\(^-\) anion exchanger; NHE: Na\(^+\)/H\(^+\) exchanger; VHA: V-type H\(^+\)-ATPase; NKA: Na\(^+\)/K\(^+\)-ATPase; MRC: mitochondria-rich cell. Note that the NHE and the VHA/Na\(^+\) channel complex are regarded as mutually-exclusive modes of Na\(^+\) uptake (\textit{i.e.}, when one is utilized, the other likely is not; Evans et al., 2005).
Figure 1.2: Schematic diagram of mechanisms for net excretion of Na⁺ and Cl⁻ ions from the blood into seawater across the fish gill epithelium. CFTR: cystic fibrosis transmembrane regulator (i.e., apical Cl⁻ channel); NKA: Na⁺/K⁺-ATPase; NKCC: Na⁺/K⁺/2Cl⁻ co-transporter; MRC: mitochondria-rich cell.
**Figure 1.3:** Schematic diagram of mechanisms for net acid excretion from the blood into the environment across the fish gill epithelium. NHE: Na⁺/H⁺ exchanger; VHA: V-type H⁺-ATPase; AE: Cl⁻/HCO₃⁻ anion exchanger; NKA: Na⁺/K⁺-ATPase; NBC: Na⁺/HCO₃⁻ co-transporter. Note that the NHE and the VHA/Na⁺ channel complex are regarded as mutually-exclusive modes of H⁺ excretion (*i.e.*, when one is utilized, the other likely is not; Evans et al., 2005).
Figure 1.4: Schematic diagram of mechanisms for net Na\(^+\) and Cl\(^-\) excretion and net acid excretion from the blood into a hypercarbic seawater environment across the fish gill epithelium. NHE: Na\(^+\)/H\(^+\) exchanger; VHA: V-type H\(^+\)-ATPase; CFTR: cystic fibrosis transmembrane regulator (i.e., apical Cl\(^-\) channel); AE: Cl\(^-\)/HCO\(_3^-\) anion exchanger; NBC: Na\(^+\)/HCO\(_3^-\) co-transporter; NKCC: Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter; NKA: Na\(^+\)/K\(^+\)-ATPase. Note that the NHE and the VHA/Na\(^+\) channel complex are regarded as mutually-exclusive modes of H\(^+\) excretion (i.e., when one is utilized, the other likely is not; Evans et al., 2005).
2 Interaction of osmoregulatory and acid-base compensation in white sturgeon (Acipenser transmontanus) during exposure to aquatic hypercarbia and elevated salinity

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2.1 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 iono- 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 (pHe), plasma [HCO\(_3\)]\(^-\), [Na\(^+\)], [Cl\(^-\)], and osmolality, white muscle water content, and gill Na\(^+\)/K\(^+\)-ATPase (NKA) and Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter (NKCC) abundance were examined over a 10-day seawater (SW) acclimation period under normocarbia (NCSW) or during prior and continued
exposure to hypercarbia (HCSW), and compared to a normocarbic 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 osmolarity over NCSW by day 2 of SW exposure. Interestingly, pHe 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.

2.2 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 is affected by other environmental stressors likely encountered during a seaward migration (such as changes in temperature and dissolved gasses). One such ‘second stressor’ likely to be encountered in this case is aquatic hypercarbia (elevated environmental PCO₂) (Cech Jr. and Doroshov, 2005), which can occur regularly in highly productive freshwater and brackish environments as a result of nighttime CO₂ 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 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 PCO\textsubscript{2} in both marine (Caldeira and Wickett, 2003) and freshwater (Sayer et al., 1993) systems increase.

In freshwater (FW) fishes, Na\textsuperscript{+} and Cl\textsuperscript{−} 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\textsuperscript{+} and Cl\textsuperscript{−} 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\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA). In seawater-acclimated fishes, Na\textsuperscript{+} 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\textsuperscript{−} is excreted transcellularly via a basolateral Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{−} 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 by a more gradual metabolic
alkalosis and recovery of blood pH (pHe) to normocapnic levels. The initial acidosis occurs as blood and tissue PCO₂ (PaCO₂) equilibrate with the increase in environmental PCO₂ (PwCO₂). In water-breathing fishes, recovery of pHe 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 (HCO₃⁻) uptake, generally in exchange with acid-base relevant ions, such as Na⁺ and Cl⁻, respectively. After dissociation of intracellular CO₂ in the presence of carbonic anhydrase (CA), H⁺ may be excreted in 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 HCO₃⁻ uptake may occur via either a basolateral Cl⁻/HCO₃⁻ anion exchanger (AE) or a basolateral Na⁺/HCO₃⁻ 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) is a particularly interesting species in which to address this question because in addition to being one of the most basal extant rayfinned 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 freshwater and may migrate to brackish waters and even full strength seawater as adults (Wilson and McKinley, 2004). Despite their known ability to tolerate seawater as adults, little is known about the development of hypoosmoregulatory 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 hours 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 PwCO$_2$) (Baker et al., 2009). At lower PwCO$_2$ tensions (1.5 kPa PwCO$_2$), white sturgeon can effectively regulate pHe, and at high (6 kPa) PwCO$_2$ tensions, they have an impressive ability to regulate intracellular pH (pHi) in most tissues despite a large depression in pHe, and thus are able to tolerate prolonged extracellular acidosis (pHe 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 seawater, and (2) acidosis compensation and osmoregulatory compensation interact on the transport of Na$^+$ and Cl$^-$.

To address these hypotheses, this study used salinity (20 ‰) and hypercarbia (6 kPa PwCO$_2$) challenges that were within the known tolerances 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 counterproductive transport of Na$^+$ (i.e., uptake for acidosis compensation vs. 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 iono- and acid-base regulatory strategy, as well as provide an interesting evolutionary context for later comparisons with the more derived teleosts.

2.3 Materials and Methods

2.3.1 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), Nanaimo, British Columbia, 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 freshwater at ~18°C under a simulated natural photoperiod for at least six months prior to experimentation, and were fed to satiation daily. The white sturgeon used here were never exposed to elevated salinity or PwCO₂ prior to this study. All procedures were performed according
to Animal Usage Protocols vetted and accepted by the Canadian Council on Animal Care (Vancouver Island University) and Institutional Animal Care and Use Committee (DePaul University).

### 2.3.2 Experimental procedure

Although sturgeon have been shown to exhibit a high tolerance and quick recovery (within 24 hours) to the physical stress of procedural handling (Baker et al., 2005; Barton et al., 2000), experimentation was designed to minimize handling stress. 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 (2,000 L) circular, darkened fiberglass tanks with recirculating filtration systems maintained at 18.5 ± 0.2 °C and well oxygenated (PwO$_2$ > 15.0 kPa). Prior to ‘day 0’ of seawater acclimation, sturgeon were exposed to either normocarbic (ambient PwCO$_2$: < 0.03 kPa) or hypercarbic (elevated PwCO$_2$: ~ 6 kPa) freshwater for 24 h. A PwCO$_2$ tension of 6 kPa was chosen to be consistent with previous hypercarbia experimentation on white sturgeon (Baker et al., 2009), and a salinity of 20 ‰ (prepared by mixing dechlorinated municipal freshwater with filtered (32 µm) local marine water (~30 ‰); referred to as ‘seawater’) was chosen to minimize osmotic stress but to ensure the requirement to hypoosmoregulate (the latter confirmed by preliminary experimentation). Implementation of hypercarbic conditions prior to seawater 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 it as a potentially confounding variable in the study.

The experimental PwCO$_2$ of 6 kPa was achieved using a PCO$_2$/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 PwCO$_2$ of 6 kPa (as inferred from pH, based upon a previously determined relationship between pH and PwCO$_2$ 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 PwCO$_2$ was returned to the desired level.

2.3.3 Sampling protocol

At 6 hours and 1, 2, 4, 7, and 10 days following seawater transfer (or in the freshwater control), eight sturgeon from each of the three control or experimental conditions were individually euthanized with MS-222 (~200 mg L$^{-1}$; buffered with NaHCO$_3$) 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 (22G) and syringe (3 mL). Blood was divided into two aliquots. The first was used to measure blood pH (considered pHe), and the second for Hct and [Hb]. Remaining blood was centrifuged (5 min at 3,000 g) and the plasma was collected and stored for measurement of TCO$_2$, 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.

2.3.4 Tissue analyses

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 manufacturer instructions; Sigma-Aldrich, St. Louis, MO, USA). Plasma TCO$_2$ was determined using a CO$_2$ analyzer (Model 965 Analyzer, Ciba-Corning Canada Inc., Markham, ON, Canada). Plasma osmolality was determined using a vapour pressure osmometer (VAPRO 5600, Wescor, Inc., Logan, UT), 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).

Intracellular pH analysis

White muscle pHi was measured using the metabolic inhibitor tissue homogenate method (validated for use in white muscle by Baker and Brauner, 2009). In this method, snap-frozen white muscle tissue was manually ground (via a liquid-nitrogen cooled mortar and pestle) into a fine powder, which was then suspended in a chilled metabolic
inhibitory cocktail of potassium fluoride (KF; 150 mM) and nitrilotriacetic acid (NTA; 8 mM) (as described and evaluated by Pörtner et al., 1990). The mixture was then transferred to a pre-cooled centrifuge tube with a pre-cooled metal scoop, then lightly vortexed and placed on ice for 10 min. pH of the mixture (considered pHi) was measured using a benchtop pH meter (Thermo Scientific Orion Benchtop pH meter, Fisher Scientific Inc., Waltham, MA, USA).

Gill Na\(^+\)/K\(^+\)-ATPase activity

Gill filaments were homogenized on ice in SEID buffer (150mM sucrose, 10mM EDTA, 50mM imidazole, and 0.1% sodium deoxycholate; pH = 7.5) by hand using a ground glass homogenizer, 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.

Na\(^+\)/K\(^+\)-ATPase 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 (PK) and lactate dehydrogenase (LDH). Gill samples were assayed for ATPase activity in the presence and absence of the NKA-specific inhibitor ouabain (1 mM). The difference in the rate of NADH oxidation (λ = 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). NKA activity was expressed as
µmol ATP · h⁻¹ · mg protein⁻¹, where protein content of the crude homogenates was determined spectrophotometrically (λ = 595 nm) as described by Bradford (1976) using a bovine serum albumin standard. All reagents listed were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Immunoblotting**

Gill tissue homogenates were prepared and protein content measured similar to those described above, differing only slightly in the homogenization buffer: 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 5 µM protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). 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) and transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were washed in PBST, blocked (in 5% nonfat milk in PBST) then incubated overnight at 4 °C in a 1:1000 dilution of the primary antibody, either mouse monoclonal anti-β-actin (as a loading control), mouse monoclonal anti-NKA α-subunit: (α5) or mouse monoclonal anti-NKCC1 (T4) (Developmental Studies Hybridoma Bank, Iowa City, IA). The use of the T4 antibody for NKCC detection in the white sturgeon gill is novel. Although the T4 antibody is known to also recognize the Na⁺/Cl⁻-cotransporter (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 hr at 23 °C in a 1:5000 dilution of a
secondary antibody (goat anti-mouse). Labeling was detected by enhanced chemiluminescence (Alpha Innotech Flour Chem HD2). Relative NKA and NKCC band intensity was analyzed using ImageJ 1.48 (National Institutes of Health, Bethesda, MD).

### 2.3.5 Calculations and statistical analyses

#### Calculations

White muscle samples were dried to a stable weight (considered ‘dry mass’) and percent water content was calculated as: \( \frac{(\text{wet mass} - \text{dry mass}) \times 100}{\text{wet mass}} \). MCHC was calculated as: \( \frac{[\text{Hb}] + \text{Hct}}{100} \). \( \text{PaCO}_2 \) and plasma \([\text{HCO}_3^-]\) were calculated from \( \text{TCO}_2 \) and pHe measurements as described by (Brauner et al., 2004), using the \( \text{CO}_2 \) solubility coefficient \( (\alpha_{\text{CO}_2}) \) and pK’ for rainbow trout (Boutilier et al., 1984) and a reorganization of the Henderson-Hasselbach equation. Differential changes in plasma \( \text{Na}^+ \) and \( \text{Cl}^- \) (\( \text{Na}^+\text{−Cl}^- \)) were calculated as \( [\text{Na}^+] - [\text{Cl}^-] \) as a means of describing differential changes in ion concentrations. Plasma \( \text{Na}^+\text{−Cl}^- \) was regressed with plasma \( [\text{HCO}_3^-] \) and a line of best fit was calculated as a means of comparing ionoregulatory status with acid-base status.

#### Statistical analyses

All values are presented as mean ± standard error of the mean (s.e.m.) \((n = 6–8)\), except for HCSW on day 10, where \( n = 4 \). Unless otherwise noted, all \( P \)-values in the text are from a two-way ANOVA analysis (used for comparison between treatments across time) with a Holm-Sidak post hoc analysis (used to identify significant differences.
between treatments at each time point). An $\alpha$-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).

2.4 Results

2.4.1 Fish health and survival

Fish transferred to seawater in both normocarbia and hypercarbia remained active and appeared in good health throughout the 10-day experiment, with the exception of the hypercarbic seawater group where 8 individuals (of the initial 54 fish in the group) became moribund between days 2–4.

2.4.2 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. 2.1); [Na$^+$] = 133 ± 1 mmol L$^{-1}$ (Fig. 2.2); [Cl$^-$] = 120 ± 1 mmol L$^{-1}$ (Fig. 2.3); and white muscle water content = 79.4 ± 0.2 % water (Fig. 2.4). Within 6 h of seawater exposure, plasma osmolality, [Na$^+$], and [Cl$^-$] in the normocarbic seawater (NCSW) sturgeon were significantly increased relative to NCFW (osmolality: $P < 0.001$; [Na$^+$]: $P = 0.010$; [Cl$^-$]: $P < 0.000$). However, by day 4, plasma osmolality, [Na$^+$], and [Cl$^-$] had recovered and were no longer significantly different from
NCNW. Changes in white muscle water content in NCSW sturgeon were consistent with changes in plasma ions, with maximal dehydration at 48 h reaching a significant difference from NCFW ($P = 0.005$). White muscle water content was also recovered and no longer significantly different after 4 d.

In 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. By 24 h after exposure to seawater, HCSW plasma osmolality, $[\text{Na}^+]$, and white muscle dehydration had increased significantly over NCFW before recovering between days 7–10. These changes were significantly (approximately two-fold) greater than the changes observed in NCSW (osmolality: $P < 0.001$; $[\text{Na}^+]$: $P < 0.001$; white muscle dehydration: $P = 0.002$). HCSW plasma $[\text{Cl}^-]$ exhibited a different pattern of change in that it did not increase above NCSW in the first 4 d, and decreased well below control values starting on day 4, reaching a significant reduction by day 10 ($P < 0.001$).

2.4.3 Acid-base balance and hematology

NCNW pHe remained stable at pH 7.62 ± 0.01 (pooled value) (Fig. 2.5). NCSW pHe exhibited a transient but significant ($P < 0.001$) acidosis after transfer to seawater, returning to NCFW values by day 4. This transient acidosis corresponded with a slight increase in plasma $[\text{HCO}_3^-]$ ($P = 0.007$) (Fig. 2.6), but was not accompanied by any significant changes in blood oxygen carrying capacity as hematocrit (Hct), hemoglobin concentration ([Hb]), and mean corpuscular hemoglobin concentration (MCHC) (Table 2.1) remained relatively stable in all normocapnic sturgeon over the 10-day
experiment. NCSW pHi (Fig. 2.7) was not different from NCFW at any sampling period despite the transient changes in pHe.

After a 24 h hypercarbia exposure (i.e., on day = 0 of seawater exposure), HCSW sturgeon had exhibited a significant extracellular acidosis ($P < 0.001$). By day 1 of seawater exposure, some compensation of pHe had occurred and was associated with increasing plasma [HCO$_3^-$], although HCSW pHe remained significantly depressed for the first week after SW transfer. By day 10, pHe in HCSW had recovered to NCFW and NCSW values. HCSW white muscle pHi did not change significantly throughout the 10-day experiment, and was not associated with pHe during recovery ($m = -0.16; r^2 = 0.49; \text{NS}$).

HCSW sturgeon did exhibit elevated Hct at day 0 ($P < 0.001$), however, this was in the absence of changes in blood [Hb], resulting in significantly lower calculated values of MCHC ($P < 0.001$). These changes were transient, and by day 10, there were no differences in Hct, [Hb], or MCHC values between experimental groups.

### 2.4.4 Gill protein activity and immunoblotting

Gill NKA activity was lowest in the NCFW control at every sampling period throughout the 10-day experiment (Fig. 2.8). NCSW NKA activity increased 4-fold during the first 7 d, significantly above NCFW ($P = 0.035$). HCSW NKA activity increased 6-fold during the first 7 d, significantly above the NCFW ($P = 0.002$) but not NCSW.

Although only a slight increase in gill NKA $\alpha$-subunit expression was observed in NCSW on day 4, there was a significant increase in expression in HCSW ($P = 0.007$).
(Fig. 2.9). NKA α-subunit expression in HCSW was nearly 3-fold that of the NCFW, and 2-fold that of the NCSW. Gill \( \text{Na}^+/\text{K}^+/2\text{Cl}^- \) cotransporter (NKCC) expression on day 4 was significantly greater (by over 2-fold) in both seawater groups compared to the NCFW control (NCSW: \( P = 0.003 \); HCSW: \( P = 0.006 \)), but expression was not different between NCSW and HCSW sturgeon (Fig. 2.10).

### 2.4.5 Plasma Na–Cl

The difference between plasma \([\text{Na}^-]\) and \([\text{Cl}^-]\) (Na–Cl) in NCFW and NCSW remained relatively stable at \( \sim 15 \text{ mmol L}^{-1} \) throughout the 10-day experiment (Fig. 2.11). In HCSW, plasma Na–Cl rose significantly above NCFW and NCSW at two distinct times, on day 1 \( (P = 0.023) \) and again on day 10 \( (P = 0.002) \). These changes in HCSW Na–Cl were significantly associated with plasma \([\text{HCO}_3^-]\) \( (m = 0.63; r^2 = 0.77; P < 0.001) \) (Fig. 2.12).

### 2.5 Discussion

#### 2.5.1 Overview

To briefly outline the following discussion with respect to the three hypotheses of this study: (1) the present study provides evidence that two-year old white sturgeon possess the hypoosmoregulatory capacity to acclimate to elevated salinity, which indicates that white sturgeon develop the ability to hypoosmoregulate between 14 and 24 months old; (2) elevated environmental PCO\(_2\) (and a resulting extracellular acidosis) appeared to increase and prolong the osmotic perturbations incurred during seawater acclimation; and
(3) Acidosis recovery appeared to only take place before and after (but not during) the period of osmotic recovery following seawater exposure. Interestingly, pHe recovery 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.

2.5.2 Normocarbic seawater acclimation in the white sturgeon

Changes in osmoregulatory status

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 Jr., 1985) white sturgeon, indicating that ‘normal’ white sturgeon plasma osmolality levels are lower than levels observed in representative freshwater teleosts (~275 mOsmol kg\(^{-1}\)) (Evans et al., 2005). When transferred to seawater, 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 hypoosmoregulating) (Gordon, 1959), then were eventually returned to a new steady state.
nearer to freshwater 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 four-day adjustment period is common among anadromous fishes; similar (or shorter) timelines have been reported in other euryhaline fishes, for example: chum salmon (12 hrs): Black, 1951; killifish (4–5 d): Marshall et al., 1999; rainbow trout (4–5 d): Leray et al., 1981.

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 seawater acclimation (Richards et al., 2003; Bystriansky et al., 2006; Bystriansky and Schulte, 2011), but is yet to be examined in sturgeon. The response in gill NKA and NKCC observed in this study supports the conclusion that two-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 hypoosmoregulate 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).

Changes in acid-base status

In addition to the osmotic disturbances seen during seawater acclimation, white sturgeon in this study also exhibit a transient change in blood pH. 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 similar to the one observed in this study (Maxime et al., 1991; Larsen and Jensen, 1992; Madsen et al., 1996). This salinity-induced acidosis is usually attributed a change in the plasma ‘strong ion difference’ status (SID; calculated as: [strong cations] – [strong anions]). In seawater-exposure studies that have included SID analysis alongside pH measurements, the metabolic acidosis observed in the first few days after salinity transfer has been associated with a decrease in SID (Maxime et al., 1991).

2.5.3 The effect of hypercarbia on seawater acclimation

Changes in osmoregulatory status

The addition of aquatic hypercarbia as a second stressor appeared to extend the adjustment period during SW acclimation—the HCSW sturgeon acclimated to seawater 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.

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 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 the white sturgeon held in freshwater (Baker et al., 2009).

Changes in acid-base status

The 24-hour exposure to aquatic hypercarbia induced an expected extracellular acidosis in the white sturgeon (i.e., the HCSW fish were in an acidotic state prior to seawater exposure). As indicated by the pH–[HCO$_3^-$] diagram (Fig. 2.6), this initial acidosis occurred along a previously determined white sturgeon non-bicarbonate buffer line (Baker et al., 2009) as PaCO$_2$ equilibrated with PwCO$_2$. Although exposure to seawater appeared to transiently aid in pHe 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 pHe, known as ‘preferential pH$i$ regulation’ (Brauner and Baker, 2009). Indeed, despite the large fluctuations in pHe, white muscle pH$i$ in HCSW remained stable, indicating that the white sturgeon in the present study
were exhibiting the preferential pH regulation. A previous study on sturgeon (Baker et al., 2009) had described a pHe recovery from a lower PwCO₂ tension within 48 h, but no pHe recovery within 48 h at PCO₂ tensions closer to that used in this study. After osmotic recovery was complete (i.e., after day = 7), the HCSW sturgeon resumed and completed a full recovery from acidosis, returning blood pH to control levels. Over the 10 days, despite there being no change in PaCO₂, the HCSW pHe recovery was associated with a continual accumulation of plasma [HCO₃⁻]. Although recovery of pHe from an acidosis of this magnitude has not yet been observed during preferential pHi regulation, 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 pHe recovery by the white sturgeon in HCSW. Further studies would be required to more specifically address this.

2.5.4 Possible interaction of osmoregulatory and acid-base compensation on net Na⁺ and Cl⁻ transport

Plasma ‘strong ion difference’ 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, as a proxy for SID, we have calculated the difference in plasma [Na⁺] and [Cl⁻] (the predominant cation and anion in the blood plasma and in the [strong cations] – [strong anions] calculation of strong ion difference) as ‘Na−Cl’. If net transport of plasma Na⁺ and Cl⁻ are equal, then the calculated plasma Na−Cl value would remain stable. Increases in the plasma Na−Cl are indicative of two potential scenarios: (1) a relative increase in plasma [Na⁺] over [Cl⁻], or (2) a relative reduction in plasma [Cl⁻] compared to [Na⁺]. Plasma Na−Cl values
presented here were positive in all sturgeon throughout the 10-day experiment, which is reflective of expectedly higher [Na\(^+\)] than [Cl\(^-\)] in the plasma compartment. Despite large fluctuations in both plasma [Na\(^+\)] and [Cl\(^-\)] during the first few days of seawater acclimation, plasma Na–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 Na–Cl (days 0–1 and 7–10). The timing of these changes corresponded to two distinct bouts of pHe recovery, and throughout the experiment, plasma Na–Cl was strongly correlated with plasma [HCO\(_3^-\)] in HCSW (but not NCFW or NCSW). These results may indicate that there is meaningful relationship between acid-base status and differential changes in plasma ions.

The increase in Na–Cl in the first 24 hours after salinity exposure (i.e., the first bout of pHe recovery) can be attributed to the HCSW sturgeon having accumulated relatively more Na\(^+\) than Cl\(^-\) during this initial salt-loading period of the seawater acclimation. The apparent association between pHe 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 Na\(^+\)/H\(^+\) exchanger or an apical H\(^+\)-ATPase with associated Na\(^+\) channel. This agrees with findings from Claiborne and Heisler (1984) and Iwama and Heisler (1991), in which elevated ambient salinity increased the rate Na\(^+\) influx and subsequent H\(^+\) efflux, resulting in a speedier pHe recovery in salinity-acclimated over freshwater-acclimated fish. Alternatively, the association of pHe recovery and Na\(^+\) accumulation may also be the result of co-transport of Na\(^+\) and HCO\(_3^-\) into the extracellular compartment via a basolateral Na\(^+\)/HCO\(_3^-\) co-transporter (NBC) (Hirata et al., 2003; Perry et al., 2003). Further investigation using pharmacological
inhibitors of these transporters would be needed to fully implicate their involvement in this process.

Although a relative increase in Na\(^+\) influx immediately following salinity exposure may have briefly aided in pH recovery, such inward transport of Na\(^+\) is counter-productive to the outward direction of Na\(^+\) transport required for seawater acclimation. As the sturgeon then made the physiological adjustments necessary to acclimate to seawater and recover plasma osmolality (presumably including a reorganization of the gill epithelium in order to excrete Na\(^+\) and Cl\(^-\)), this may have been to the detriment of any further pH recovery. This might explain the plateau of pH recovery during days 1–7 after seawater exposure, as well as the return of plasma Na–Cl nearer to control values during this time. This stagnation of pH recovery may also imply a prioritization of osmoregulatory recovery over acid-base recovery in this period of combined exposure to acid-base and osmoregulatory disturbances.

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

This study has described the time course of seawater acclimation by juvenile white sturgeon. The two-year-old age class in the present study represents the youngest age class of white sturgeon examined to date which has been able to make the physiological adjustments necessary to hypoosmoregulate in elevated salinity. Simultaneous exposure to elevated dissolved CO$_2$ significantly increases the osmotic disturbance incurred during acclimation to seawater. Exposure to elevated dissolved CO$_2$ also affects the ionic composition of the blood plasma during seawater acclimation, and these differences in ionic composition appear to be related to acid-base status. The potential associations of relative plasma Na$^+$ accumulation and relative plasma Cl$^-$ removal with pHe recovery substantiate similar findings from other investigations into the relationship between Na$^+$ and Cl$^-$ transport and acid-base balance. Unique to this study is a description of blood pH recovery in hypercapnic sturgeon associating more prominently with plasma Na$^+$ accumulation prior to osmotic recovery and more prominently with plasma Cl$^-$ removal following osmotic recovery, as the requirements of osmoregulation transitions from ion uptake to ion excretion throughout seawater acclimation.
2.6 Figures

**Figure 2.1**: Plasma total osmolality in white sturgeon acclimated to freshwater (circles) or seawater (20 ‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; $P < 0.05$): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.2: Plasma sodium in white sturgeon acclimated to freshwater (circles) or seawater (20 ‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; P < 0.05): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.3: Plasma chloride in white sturgeon acclimated to freshwater (circles) or seawater (20‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; *P < 0.05): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
**Figure 2.4:** White muscle water content in white sturgeon acclimated to freshwater (circles) or seawater (20 %o, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; \( P < 0.05 \)): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.5: Blood pH in white sturgeon acclimated to freshwater (circles) or seawater (20‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; $P < 0.05$): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.6: Changes in acid-base status in white sturgeon during acclimation to seawater (20 ‰) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂. Blood pH is plotted against plasma [HCO₃⁻]. PaCO₂ isopleths (solid lines) and the blood non-bicarbonate buffer line (dotted line; adapted from Baker et al., 2009) are included for reference. Values presented as mean ± s.e.m. Numbers on graph indicate day of acclimation.
Figure 2.7: Relationship between blood pH (pHe) and white muscle intracellular pH (pHi) in white sturgeon during acclimation to freshwater (circles) or seawater (20 ‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO₂ over 10 days. Values presented as mean ± s.e.m. The solid line ($m = -0.16; r^2 = 0.49; NS$) describes the correlation between pHe and white muscle pHi in hypercarbic seawater (HCSW) sturgeon. Numbers on graph indicate day of acclimation in HCSW group.
Figure 2.8: Gill Na\(^+\)/K\(^+\)-ATPase (NKA) activity in white sturgeon during acclimation to freshwater (circles) or seawater (20‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO\(_2\). Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; \(P < 0.05\)): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
**Figure 2.9:** Effects of acclimation to normocarbic freshwater (NCFW), normocarbic seawater (NCSW; 20‰), or hypercarbic seawater (HCSW; ~6 kPa PwCO₂) on protein abundance (A) of gill Na⁺/K⁺-ATPase α-subunit (110 kDa) and β-actin (42 kDa) in the white sturgeon via Western blot. Values in B are presented relative to a β-actin loading control as mean protein abundance ± s.e.m. Symbols denote statistically significant differences (two-way ANOVA; *P* < 0.05): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.10: Effects of acclimation to normocarbic freshwater (NCFW), normocarbic seawater (NCSW; 20 ‰), or hypercarbic seawater (HCSW; ~6 kPa PwCO₂) on protein abundance (A) of gill Na⁺/K⁺/2Cl⁻ co-transporter (NKCC) (225 kDa) in the white sturgeon via Western blot. Values in B are presented relative to a β-actin loading control (shown in Fig. 2.9) as mean protein abundance ± s.e.m. Symbols denote statistically significant differences (two-way ANOVA; P < 0.05): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.11: Difference in plasma $[\text{Na}^+]$ and $[\text{Cl}^-]$ (Na−Cl; calculated as: $[\text{Na}^+] - [\text{Cl}^-]$) in white sturgeon acclimated to freshwater (circles) or seawater (20 ‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO$_2$. Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; $P < 0.05$): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
Figure 2.12: Relationship between difference in plasma \([\text{Na}^+]\) and \([\text{Cl}^-]\) (Na–Cl; calculated as: \([\text{Na}^+] - [\text{Cl}^-]\)) and plasma \([\text{HCO}_3^-]\) in white sturgeon acclimated to freshwater (circles) or seawater (20 ‰, squares) under ambient (white fill) or elevated (~6 kPa; black fill) PwCO\(_2\) over 10 days. HCSW correlation is described by solid line \((m = 0.59; r^2 = 0.58; P < 0.001)\). Dotted line displays the line of identity \((m = 1)\).
Table 2.1: Experimentally determined values for hematocrit (% packed cell volume), hemoglobin concentration (g dL$^{-1}$), and calculated mean corpuscular hemoglobin concentration (g dL$^{-1}$) in white sturgeon acclimated to either normocarbic freshwater (NCFW), normocarbic seawater (NCSW; 20 %), or hypercarbic seawater (HCSW; ~6 kPa PwCO$_2$) over 10 days.

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Values presented as mean ± s.e.m. Symbols denote statistically significant differences at respective time point (two-way ANOVA; $P < 0.05$): * denotes difference from normocarbic freshwater control; + denotes difference between seawater treatments.
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