Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*

D. W. Baker,1 V. Matey,2 K. T. Huynh,1 J. M. Wilson,3 J. D. Morgan,4 and C. J. Brauner1

1Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada; 2Department of Biology, San Diego State University, San Diego, California; 3Center for Marine and Environmental Research (CIMAR), Porto, Portugal; and 4Faculty of Science and Technology, Vancouver Island University, Nanaimo, British Columbia, Canada. Submitted 12 September 2008; accepted in final form 29 March 2009

Baker DW, Matey V, Huynh KT, Wilson JM, Morgan JD, Brauner CJ. Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*. Am J Physiol Regul Integr Comp Physiol 296: R1868–R1880, 2009. First published April 1, 2009; doi:10.1152/ajpregu.90767.2008.—Sturgeons are among the most CO2 tolerant of fishes investigated to date. However, the basis of this exceptional CO2 tolerance is unknown. Here, white sturgeon, *Acipenser transmontanus*, were exposed to elevated CO2 to investigate the mechanisms associated with short-term hypercarbia tolerance. During exposure to 1.5 kPa PCO2, transient blood pH [extracellular pH (pHe)] depression was compensated within 24 h and associated with net plasma HCO3− accumulation and equimolar Cl− loss, and changes in gill morphology, such as a decrease in apical surface area of mitochondrial-rich cells. These findings indicate that pHe recovery at this level of hypercarbia is accomplished in a manner similar to most freshwater teleost species studied to date, although branchional mechanisms involved may differ. White sturgeon exposed to more severe hypercarbia (3 and 6 kPa PCO2) for 48 h exhibited incomplete pH compensation in blood and red blood cells. Despite pHe depression, intracellular pH (pHi) of white muscle, heart, brain, and liver did not decrease during a transient (6 h of 1.5 kPa PCO2) or prolonged (48 h at 3 and 6 kPa PCO2) blood acidosis. This pHi protection was not due to high intrinsic buffering in tissues. Such tight active cellular regulation of pH in the absence of pHe compensation represents a unique pattern for non-air-breathing fishes, and we hypothesize that it is the basis for the exceptional CO2 tolerance of white sturgeon and, likely, other CO2 tolerant fishes. Further research to elucidate the specific mechanisms responsible for this tremendous pH regulatory capacity in tissues of white sturgeon is warranted.

Hypocapnia in fish; acid-base regulation; intracellular pH; CO2 tolerance; hypercapnia/hypercarbia

AQUATIC HYPERCARBIA (elevated PCO2 in water) occurs in fresh and estuarine systems, and PWCO2 levels as great as 8 kPa (20- to 30-fold increase over the resting arterial PCO2 of fish) have been observed (24, 25) in tropical freshwater environments. Exposure to elevated aquatic PCO2 induces physiological and morphological changes that have been reasonably well described in a few species of teleost [e.g., rainbow trout, *Oncorhynchus mykiss* (31, 57); common carp, *Cyprinus carpio* (13); brown bullhead, *Ictalurus nebulosus* (20)] and elasmobranch [e.g., *Scyllorhinus stellaris* (26); *Raja ocellata* (21, 58)] fishes. The initial rapid respiratory acidosis is corrected by a more gradual metabolic alkalosis, which may, over hours or days, return pH in blood and extracellular fluid (together referred to hereafter as pHe) to preexposure levels [i.e., pHe at normocapnic or resting arterial PCO2 (PACO2)]. This pHe recovery is achieved primarily at the gill [80−95%, (43, 45)], typically via alterations in net plasma HCO3− and Cl− concentrations (33, 57), although other acid-base relevant counter ions may be important [e.g., Na+(21, 23, 27)]. Additionally, hypercarbia induces morphological [e.g., reduced apical surface area of mitochondrial-rich cells (MRC) and increased surface area of pavement cells (PVC) (20)] and molecular [changes in activity or expression of, for example, the proton exchangers (NHE3) (16)] changes at the gill. These changes in branchial morphology and acid-base-relevant ion transporters may aid with the net acid secretion or base absorption mechanisms necessary to promote blood pH compensation (16, 20), although direct evidence for this is lacking.

Changes in intracellular pH (pHi) in most fish species studied to date are qualitatively similar to, albeit smaller than, blood pH changes during a respiratory acidosis (6, 49). Because function of many cellular components, such as enzyme activity, is pH sensitive, a general acidosis may have severe consequences on cellular processes, including metabolic energy production (25, 50). Only a handful of studies have measured pHi and pHe simultaneously during hypercarbia in fish; these studies indicate that most tissues, such as heart, white muscle, and liver, recover pHi in proportion to pHe (for a review, see Ref. 6), although a few instances of more rapid tissue pHi recovery exist [e.g., gill in rainbow trout, (57); brain in *R. ocellata* (58)].

As hypercarbia and the resulting acidosis increase in severity, complete pHe recovery becomes limited due to the bicarbonate concentration threshold (6, 25). During short-term exposure (days) to hypercarbia, most fish species studied to date are not capable of increasing plasma HCO3− (in exchange with Cl−) >27−33 mmol/l, and thus cannot fully compensate for the respiratory acidosis associated with CO2 levels greater than ~2 kPa PCO2 (15 Torr PCO2, where 1 Torr equals 0.133 kPa) (6, 25). This failure is associated with mortality, although surprisingly little research has described CO2-related toxicity (23, 27). Despite this pHe compensatory constraint, a few fish species [e.g., European eel, *Anguilla anguilla* (37)] are able to tolerate exposures much greater than 2 kPa PCO2 in the face of prolonged pHe depression. Two of these tolerant species, the facultative air breathers, *Synbranchus marmoratus* (24) and *Pterygoplichthys* (formerly *Liposarcus*) *pardalis* (8) have demonstrated an ability to protect pHi in some tissues (e.g., heart
and white muscle) during a prolonged and severe blood acidosis. Consequently, we hypothesized that exceptional hypercarbia tolerance in fish is associated with the ability to tightly regulate pH to tissues during a transient or prolonged uncompensated respiratory acidosis.

White sturgeon (*Acipenser transmontanus*, Richardson, 1836) can tolerate levels of hypercarbia that induce a severe blood acidosis for days (14). The objective of this study was to investigate acid-base regulation in white sturgeon during hypercarbic challenges both within (1.5 kPa PCO₂) and beyond (3 and 6 kPa PCO₂) the pH compensatory capacity of most fishes. To elucidate the mechanisms of pH compensation during hypercarbia, we measured changes in blood physiology (e.g., PHe, [HCO₃⁻] and [Cl⁻]), gill morphology (e.g., apical surface area of MRC), and branchial acid-base-relevant transporter (e.g., V-ATPase) activity and expression induced by 1.5 kPa PCO₂.

To test our hypothesis that white sturgeon protect tissue pH during an extracellular acidosis, we characterized pH changes in tissues (including red blood cells [RBC], heart, liver, brain, and white muscle) in response to the hypercarbic challenges described above. To further elucidate the role of active pH regulatory mechanisms involved in pH regulation, we calculated intracellular intrinsic (i.e., nonbicarbonate) buffer values from homogenized white sturgeon tissues (i.e., white muscle, heart, and liver). Since sturgeon represent one of the most basal extant actinopterygians (28) they have enormous value for studying vertebrate evolution (10) and may provide further insight into the basis of hypercarbia tolerance in fishes.

**METHODS**

*Animal acquisition and holding.* White sturgeon, *A. transmontanus*, for all experiments were progeny of wild-caught brood stock (which has been spawned successfully since 1991) from Vancouver Island University (VIU; formerly Malaspina University College) in Nanaimo, BC, Canada. Experiments in series 1 (see below) were performed at VIU in the fall with 3-yr-old white sturgeon (length ~50–80 cm, mass ~1–2.5 kg), where water was very soft and dilute (hardness: 12 μmol/l [CaCO₃], alkalinity: 13–14 μmol/l, pH: 6.6–6.9, [Na⁺] and [Cl⁻] < 1 μmol/l each). For series 2, white sturgeon (4 yr old, mass ~1–2 kg) initially spawned at VIU were obtained from Target Marine Hatchery (Sechelt, BC, Canada) and held at the University of British Columbia (UBC), Vancouver, BC, for several months prior to experimentation. Water at this facility was even softer (water hardness: 4 μmol/l [CaCO₃], alkalinity: 3–4 μmol/l [CaCO₃], pH: 6.7–7.0, [Na⁺] and [Cl⁻] < 3 μmol/l each).

All animals were held in large, outdoor flow-through tanks (PwO₂ > 15.0 kPa, PwCO₂ < 0.03 kPa, T = 11–17°C, fish density < 25 kg·fish·m⁻³ water) and fed a commercial diet to satiation daily prior to experiments. No mortality occurred during transport, holding, or exposure to any CO₂ levels used in this study in the 3-mo period prior to terminal sampling. Both series of experiments were performed in the fall to reduce seasonal variability. Food was withheld 24 h prior to experimentation. All holding and experimental protocols were approved by the Animal Care Committees at UBC (animal usage protocol no. AC 02–0222) and VIU (animal usage protocol no. 2004–04(R)).

**Series 1: effect of exposure to 1.5 kPa PCO₂ on blood and tissues of white sturgeon.** White sturgeon were placed into a recirculating system (PwO₂ > 15.0 kPa, and PwCO₂ < 0.03 kPa PCO₂), consisting of darkened plastic boxes (30 liters, flow rate ~3 l/min, 17°C), for 24 h prior to experiments. This period is sufficient to allow recovery from handling stress in sturgeon (e.g., 2, 3, 15). Normocarbic white sturgeon were terminally sampled immediately following this habituation period (control or preexposure group). Sturgeon were then exposed to either a further 48 h of normocarbia, or to PwCO₂ ~1.5 kPa PCO₂ for 6, 24, or 48 h, which was induced by pluming a mixing tank into the recirculating system and bubbling it with preset rates of air and 100% CO₂ using Sierra Instruments mass flow controllers. PwCO₂ was measured with a Pco₂ electrode to confirm target CO₂ tensions. Water oxygen levels remained high throughout all treatments (PwO₂ > 14 kPa).

Following exposure, each box was isolated from the recirculation system, and the animals were euthanized with MS-222 (0.3 g/l, buffered with NaHCO₃). After ventilation ceased (<5 min), each fish was immediately transferred to a surgery table and blood (3 ml) was drawn caudally via a sterile lithium-heparin (1 g/l)-rinsed syringe (10-ml syringe, 25-G1 needle), and placed on ice. Following this procedure (<1 min), fish were killed and the following tissues were excised, placed in prelabeled tissue, and immediately freeze-clamped with liquid nitrogen-cooled tongs in this order: liver (1 g), heart, brain, dorsal white muscle (1 g, skin and red muscle removed) and second and third gill arches (left side). All freeze-clamped tissues were then stored at ~80°C. Next, second and third gill arches (right side) were removed and stored in either Karnowsky’s solution for electron microscopy (second arch), or 3% paraformaldehyde in PBS for immunofluorescence microscopy (third arch). Blood was divided into two equal aliquots. Hemoglobin concentration ([Hb]), hematocrit (Hct) and mean cell hemoglobin concentration (MCHC) were measured from 1 aliquot, and from the other, blood pH (PHe), plasma total carbon dioxide (TCO₂), and plasma ions (Na⁺, Cl⁻, Mg²⁺, and Ca²⁺) were measured, as described below.

**Series 2: effect of exposure to 3 and 6 kPa PCO₂.** White sturgeon were anaesthetized (MS-222, 0.2 g/l, buffered with NaHCO₃), transferred to a surgical table, and, while gills were irrigated with an oxygenated MS-222 (0.05 g/l buffered with NaHCO₃) solution, a dorsal aortic catheter (PE-50; Intramedic) was surgically implanted as has been previously described (14). Following surgery, each cannulated sturgeon was transferred to a black box (30 liters) supplied with recirculated aerated water (flow rate: >3 l/min, T = 13°C). Each cannula was flushed daily and following sampling with lithium-heparin (0.2 g/l) Cortland’s saline.

Following 36-h recovery in normocarbia from surgery, a blood sample (400 μl) was drawn from the cannula into a heparinized 1-ml syringe, placed on ice, and PHe, plasma [HCO₃⁻], [Hb], Hct, MCHC, and [Cl⁻] were measured as described below. RBC pH was measured when volume permitted. Fish were then exposed to water equilibrated with one of three CO₂ tensions: 1) normocarbic water (air saturated), 2) 3 kPa PCO₂, or 3) 6 kPa PCO₂. Water PCO₂ was verified via a thermostated (13°C) Radiometer PCO₂ electrode (E5036) (output, Radiometer PHM 73). Blood samples (300 μl) were taken from each fish at 0, 15, and 30 min, and 1, 3, 6, 12, 24, and 48 h. After 48 h, white sturgeon were terminally anaesthetized as described in series 1, and brain, heart, liver, and white muscle were surgically excised, freeze-clamped, and stored for later measurement of tissue pH and nonbicarbonate buffering.

**Analytical techniques.** [Hb] (using Drabkin’s reagent), Hct, and RBC MCHC were determined as described previously (2, 3). Blood glucose was measured with a blood glucose meter (Ascensia Elite; Bayer). Blood pH was measured using a thermostated capillary pH electrode (model MBS 3 MK 2; Radiometer). The remaining blood was centrifuged (3 min at 10,000 rpm), and plasma was removed for measurement of TCO₂ (model 965 Analyzer; Corning), osmolality (model 5520; Westcor Vapor Pressure Osmometer), and inorganic ions ([Na⁺] model 410 Flame photometer; Corning; [Cl⁻] HBI model 442500; digital chlorideometer, [Ca²⁺] and [Mg²⁺], model AA 240 FS, flame spectrophotometer; Varian). Blood Pco₂ and plasma [HCO₃⁻] were calculated from total CO₂ and pH measurements as described previously (8), using the CO₂ solubility coefficient (αCO₂) and pKₐ for rainbow trout (5) and a reorganization of the Henderson-Hasselbalch equation. This indirect method has been used previously for fish exposed to hypercarbia (8) but assumes Pco₂ is equilibrated.
between blood and tissues, which may not be the case in vivo. At higher CO$_2$ tensions, however, potential PCO$_2$ differences due to incomplete equilibration are relatively small, and thus would have little impact on this calculation. Separated RBC pellets were analyzed for pH using the freeze-thaw technique (38, 59). Tissues were later ground under liquid nitrogen, and pH was measured using the metabolic inhibitor tissue homogenate method (48), which we have verified to be accurate in tissues exposed to higher PCO$_2$ tensions (5). Tissue [HCO$_3^-$] was calculated as in blood, using pK’ values from previous research (5), and assuming PCO$_2$ to be in equilibrium between water, blood, and tissues, as has been assumed in previous work (5, 24, 36). RBC and tissue pH was measured using the same thermostatted electrode as that described above for blood.

Nonbicarbonate whole blood buffer capacity was determined on caudally sampled blood transferred to Eschweiller thermostated (13°C) glass tonometers (4 ml each), and equilibrated for 45 min at 0.5, 1, 2, 4, 6, or 10 kPa PCO$_2$ using a Wösthoff (DIGAMIX 6KM 422) gas mixing pump (13°C) glass tonometers (4 ml each), and equilibrated for 45 min at caudally sampled blood transferred to Eschweiller thermostated electrode as that described above for blood. (5, 24, 36). RBC and tissue pH was measured using the same thermostatted electrode as that described above for blood.

Immunoblotting. Immunoblotting was carried out as described previously (56). Briefly, samples were electrophoretically separated by SDS-PAGE (10%,T), and transferred to PVDF membranes by semidy electrophoretic transfer. Membranes were then blocked with 5% blotto in 0.05% Tween-20 in Tris-buffered saline, pH 7.4 (1 h) and probed overnight with primary antibody diluted 1:1,000 [mouse monoclonal anti-Na$^+$-K$^+$-ATPase α-subunit: clone α5; rabbit polyclonal anti-V-ATPase B subunit: B1/B2VATP (56) or rat polyclonal anti-NHE3: R1B2 (12) antibodies]. Following washes in Tween-20...

![Fig. 1. Effect of exposure to hypercarbia (1.5 kPa or 11 Torr PCO$_2$) for 6, 24, and 48 h on arterial pH and plasma [HCO$_3^-$] (meq/l) presented as a pH/bicarbonate/CO$_2$ diagram (A), red blood cell (RBC) intracellular pH (pHi; B), and brain (○), liver (■), and heart (●) pH (C) in white sturgeon, Acipenser transmontanus. Values are presented as means ± SE. (n = 6–7). A: time in hours is indicated next to each point (0, 6, 24, 48); dashed line = blood nonbicarbonate buffer line; *significant change in pH; †significant change in pH; ● significant change in plasma [HCO$_3^-$] from the normocarbic group (control). B and C: *significant difference from the normocarbic group (control).]
Tris-base sodium, membranes were incubated with goat anti-rat, mouse, or rabbit horseradish peroxidase-conjugated secondary antibody (1:100,000), and labeling was detected by enhanced chemiluminescence (Immobilon; Millipore) using a charge-coupled device camera imaging system (model LAS 4000mini; Fujifilm, Tokyo Japan). Bands were quantified using Fujifilm Science Lab software.

**Results**

Series 1: effect of exposure to 1.5 kPa Pco2 on blood and tissues of white sturgeon. White sturgeon exposed to 1.5 kPa Pco2 exhibited an increase in blood Pco2 (2.9 ± 0.5 to 11.6 ± 0.4 Torr, P < 0.05, Fig. 1A). Blood pH decreased significantly in these fish after 6 h; after 24 h, it was no longer significantly different from control fish (Fig. 1A). Mean plasma [HCO3−] was significantly higher (Fig. 1A), and mean plasma [Cl−] was significantly lower than time 0 following 6, 24, and 48 h of this CO2 exposure (Table 1). Plasma [Na+], [Mg2+], and osmolality did not change significantly, although plasma [Ca2+] was significantly higher following hypercarbica exposure (Table 1). [Hb] (pooled value: 0.85 ± 0.025 mmol/l), Hct (pooled value: 33.2 ± 0.9%), and MCHC (pooled value: 2.53 ± 0.024 mmol/kg packed RBCs) of hypercarbic sturgeon were not significantly different from either preexposed sturgeon or sturgeon exposed to 48 h of normocarbia. Blood glucose of hypercarbic fish was significantly higher at 6 h compared with 48 h (Table 1). RBC pH i was significantly depressed following 6 h, but not 24 or 48 h (Fig. 1B); in fact, at 48 h, pH i was significantly elevated. White muscle pH i did not change significantly (pooled value: 7.20 ± 0.02), although white muscle [HCO3−] was elevated at 24 and 48 h (Table 2). Brain, liver, and heart pH i (Fig. 1C) and [HCO3−] (Table 2) were significantly elevated over respective control tissue values following 6, 24, and 48 h of exposure. Blood pH (pHe) was significantly correlated with RBC pH i (slope = 0.48, r² = 0.26, P < 0.05), but not with white muscle, brain, heart, or liver pH i.

In control fish, PVC comprised ~90% of the filament epithelium surface with MRC accounting for 10%. PVC apical surfaces displayed a complex pattern formed by short and long microridges, with cellular borders not clearly defined (Figs. 2B and 3A). The apical surface of the MRC varied in appearance,
size, and topography, such that there appeared to be two distinct populations. Apexes of ~67% of MRC were large, slightly convex, and ornamented with either long, thin, and ramified microvilli or shorter and thicker microvilli [referred to as MRC with a larger surface area (MRCLA), Figs. 2, B–D, 3A]. The rest of the MRC with a smaller surface area (MRCSA) exhibited small, mostly circular, flat or slightly convex apical surface with short microvilli (Fig. 3A).

Exposure to 1.5 kPa PCO₂ caused morphological modifications in both PVC and MRC. In PVCs, the most complex “lace-like” patterns and, correspondingly, the highest density of branched and interdigitated microridges were observed after 6 h of hypercarbia (Figs. 3B and 4A). After 48 h, microridge density was no longer significantly higher than control (Figs. 3D and 4A). Cellular borders between PVC became clearly defined with tall parallel microridges (Fig. 3D). Density and total surface area of MRCLAs was significantly reduced during this CO₂ challenge (Figs. 3, B–D, and 4, B–D). After 6 h, density of MRC decreased almost 30% below control (Fig. 4B), and the fractional area of MRC decreased to less than half that of control values (Fig. 4C). The surface area of individual MRC at 6 h was significantly lower than in normocarbic gills and decreased further by 24 h of exposure to 1.5 kPa PCO₂ (Fig. 4C).

Na⁺-K⁺-ATPase activity increased significantly after 48-h exposure to 1.5 kPa PCO₂ (Fig. 5A); however, Western blot analysis indicated Na⁺-K⁺-ATPase α-subunit protein levels did not change, as indicated by representative blots (Fig. 5C). A single band of ~100 kDa was identified in gill homogenates. V-ATPase activity and B subunit protein levels were unaffected (Fig. 5, B and D). A single band of ~56 kDa was recognized by the B1/B2VATP polyclonal antibody. The NHE3 R1B2 antibody weakly cross reacted with bands at ~75 and ~50 kDa (Fig. 5E), but no changes in either band were observed during exposure to 1.5 kPa PCO₂.

In gills from normocarbic sturgeon, the Na⁺-K⁺-ATPase α-subunit was immunolocalized to cells in the interlamellar and lamellar epithelia (Fig. 6, A and D). Labeling of these cells was either restricted to the basolateral membrane or throughout the cell body (excluding the nucleus). NHE3 was immunolocalized to the apical membrane of a subpopulation of these cells as well as some non-Na⁺-K⁺-ATPase immunoreactive cells (Fig. 6, B and C). There was also some weaker cytoplasmic staining in these cells. In general, the NHE3 immunoreactivity was weak, which required longer exposure times, and thus background staining was more of a problem. The labeling of erythrocytes, which was nonspecific as determined by control staining, was particularly noticeable. V-ATPase immunofluorescence was generally found in cells without detectable Na⁺-K⁺-ATPase immunoreactivity; in some cells, however, there was colocalization of staining (Fig. 6, D–F). V-ATPase immunoreactivity cells were found in both the filament and lamellar epithelia and generally had a cytoplasmic distribution, although in some cases, staining was limited to the apical region of these cells. Qualitative observations of staining patterns for Na⁺-K⁺-ATPase, NHE3, and V-ATPase did not change appreciably with 48 h of exposure to 1.5% CO₂.

Series 2: effect of exposure to 3 and 6 kPa PCO₂ on blood and tissues of white sturgeon. Normocarbia-exposed sturgeon exhibited no significant changes in pHe, plasma [HCO₃⁻] or plasma [Cl⁻] over the course of the 48-h experiment. Within 15 min of exposure to 3 and 6 kPa PCO₂, pHe decreased to remain significantly different throughout the 48-h exposure, although some pHe recovery did occur within each treatment compared with the lowest pH values measured at 3 h (Fig. 7A). Plasma [HCO₃⁻] was significantly elevated at 1 h compared with time 0, and remained so throughout exposure to both 3 and 6 kPa PCO₂ (Fig. 7B). Plasma [Cl⁻] decreased significantly after 24 h of exposure to 3 (141 ± 4.3 to 131 ± 4.1 mmol/l) and 48 h of exposure to 6 kPa PCO₂ (152 ± 6.3 to 123 ± 5.0 mmol/l). White sturgeon Hct (pooled value 28.8 ± 0.4%) and MCHC (pooled value 2.63 ± 0.04 mmol/l packed RBC) did not change significantly over the course of the experimental exposures, although a trend
of decreasing mean [Hb] and Hct was observed in all groups, likely the result of repeated blood sampling.

Red blood cell pH was significantly depressed in fish exposed to hypercarbia at all time points compared with RBC pH of normocarbia-exposed fish (Fig. 8A), despite some pH recovery after 48 h. When pH was regressed against RBC pH over the course of the experiment (Fig. 8B), or at 48 h (Fig. 9A), a statistically significant positive correlation was found. However, mean pH of heart, liver, brain, and white muscle of white sturgeon was not significantly different between treatments, despite the substantial difference in pH between treatments (Fig. 8). Furthermore, pH, regressed against pH in heart, brain, liver, and white muscle after 48 h of hypercarbia, did not exhibit the positive relationship observed for RBC pH (Fig. 9, A and B). Tissue [HCO₃⁻] was elevated following 48 h of both CO₂ treatments (Table 2).

Increasing CO₂ levels altered both blood (Fig. 10) and tissue water (Fig. 11, A–C) pH and [HCO₃⁻]. Blood β₂NB was −11.9 mmol HCO₃⁻·pH unit⁻¹·l blood⁻¹ (r² = 0.88). Nonbicarbonate (i.e., intrinsic) buffer capacity in white muscle, heart, and liver of white sturgeon (as calculated from Fig. 11, A–C over an in vivo relevant pH and CO₂ range) was 35.3, 11.3, and 8.9 mmol HCO₃⁻·pH unit⁻¹·kg intracellular tissue water⁻¹, respectively.

DISCUSSION

White sturgeon responded to a respiratory acidosis induced by exposure to 1.5 kPa pCO₂ with pH compensation qualitatively similar to most other fishes examined (e.g., 9, 33, 44). Changes in gross gill morphology were also similar to what has been observed in teleosts (20), although patterns of activity and expression of branchial ionoregulatory transporters during hypercarbia were different in white sturgeon compared with other teleosts studied (16, 17). When exposed to 3 and 6 kPa pCO₂, pH recovery was incomplete and associated with limited net HCO₃⁻ accumulation. However, pH was preferentially regulated in the heart, liver, brain, and white muscle (but not RBC) to normocapnic pH levels during hypercarbia, despite either transient or extended pH depression. This pH homeostasis is beyond what could be attributable to intrinsic (i.e., nonbicarbonate) intracellular buffering, and therefore is most likely due to active transmembrane acid-base relevant ion transport at the cellular level. This active pH regulatory capacity, particularly
in the brain, liver, and heart, likely represents the basis for the exceptional tolerance of sturgeon to short-term CO2 exposure.

White sturgeon during normocarbia. Blood and RBC physiological parameters for normocarbic white sturgeon in this study fell within the range of values reported previously for North American sturgeons (e.g., 2, 3). Most values from normocarbic white sturgeon tissues in this study were consistent with that observed in teleosts [e.g., cod, Gadus morhua (32), P. pardalis, (8), S. marmoratus (24), and rainbow trout (57)], although there were a few discrepancies [e.g., rainbow trout (58) and sea raven (Hemitripterus americanus, 39) hearts]. Brain pHi has rarely been measured in fishes, but values for normocarbic trout (57) are higher (0.2–0.3 pH units) than values for the white sturgeon in this study.

pHe recovery in white sturgeon. This study assessed the capacity of white sturgeon to alter net epithelial acid-base relevant ion transport to drive pH recovery during hypercarbia (1.5 kPa). Changes in white sturgeon acid-base physiology during a moderate hypercarbic challenge (1.5 kPa PCO2 over 48 h) were qualitatively similar to those reported in most teleosts (e.g., 20, 57) and elasmobranchs (e.g., 21, 26) investigated to date. White sturgeon exhibited rapid recovery associated with a net elevation in plasma [HCO3−] matched by an

Fig. 4. Effect of exposure to hypercarbia (1.5 kPa PCO2) for 6, 24, and 48 h on PVC micridge density (intercepts/grid) (A), MRC density (mm−2) (B), MRC surface area (μm2) (C), and MRC fractional area (% epithelium/unit) (D) on the filament epithelium in the white sturgeon. Values are presented as means ± SE (n = 6–7). a,bSignificant differences between groups.

Fig. 5. Effect of exposure to hypercarbia (1.5 kPa PCO2) for 6, 24, and 48 h on activity of either branchial Na+/K+-ATPase activity (μmol ADP/mg protein) (A), V-type H+-ATPase (V-ATPase) activity (μmol ADP/mg protein) (B), or expression of α-subunit of Na+/K+-ATPase (C), B subunit of V-ATPase (D), or NHE3 in representative Western blots (E) in white sturgeon. A and B: values are presented as means ± SE. (n = 6–7). **Significant differences.
equimolar reduction in plasma \( [\text{Cl}^-] \). The time course of \( \text{pHe} \) recovery was similar, if perhaps more rapid than that observed in rainbow trout exposed to a similar \( \text{CO}_2 \) challenge (31).

Blood \( \text{pH} \) compensation at 1.5 kPa \( \text{PCO}_2 \) was associated with significant alterations in the apical surface morphology of both PVC and MRCs. For example, the apical surface of the PVC that directly interacts with ambient water became more ruffled due to an increase in the density of ramified and interdigitated microridges (Figs. 3 and 4A). These ridges greatly increase the surface area of these cells and may reflect high functional activity (e.g., the number of sites available for proton excretion, 20). Concurrently, the fractional surface area of MRCl exhibited a progressive reduction: after 48 h, this decrease was > 50% (Figs. 3 and 4D). This was due to all of the following: 1) a decrease in the number of apically exposed MRC (Fig. 4B), 2) morphological alteration of MRCl to MRCSA, and 3) reduction of surface area of MRC (Fig. 4C). As MRC may be the most important site of chloride uptake in freshwater fishes (17, 20), these changes, which have also been observed in teleosts exposed to hypercarbia (20), are hypothesized to reduce sites of chloride uptake or base excretion (17, 20). \( \text{Na}^+\text{-K}^+\text{-ATPase} \), heavily concentrated on the basolateral membrane of MRC, exhibited increased activity during hypercarbia (Fig. 4A), and, as \( \text{Na}^+\text{-K}^+\text{-ATPase} \) was found colocalized with \( \text{V-ATPase} \) in some branchial sites (Fig. 6, D–F), this finding could imply a contribution of this enzyme to branchial or whole animal \( \text{pH} \) homeostasis.

In contrast, exposure to 1.5 kPa \( \text{PCO}_2 \) did not induce changes in NHE3 protein levels or either \( \text{V-ATPase} \) activity or \( \text{V-ATPase} \) B subunit expression in white sturgeon (Fig. 5D). In rainbow trout, \( \text{O. mykiss} \), increases in \( \text{V-ATPase} \) activity and expression have been observed in response to hypercarbia [NEM-sensitive proton \( \text{ATPase} \) activity (34); \( \text{V-ATPase} \) E subunit expression in immunoreactive cells (52); \( \text{V-ATPase} \) A subunit expression in immunoreactive cells, (35)]. The lack of response in sturgeon may be related to the time course of these experiments, as 48 h may not have been sufficient for changes in activity to occur. However, changes in both concentration and activity of these transporters have been demonstrated to occur within this time frame in other studies (34, 52). It is also possible that existing transporters are sufficient to drive \( \text{pHe} \) compensation, as increases in NHE3 expression are not always seen in response to hypercarbia (e.g., Atlantic stingray, \textit{Dasyatis sabina} (11); freshwater-acclimated killifish, \textit{F. heteroclitus} (16)). A third possibility is that NHE3 and \( \text{V-ATPase} \) play a limited role in branchial \( \text{pH} \) compensation of white sturgeon to hypercarbia. If this were the case, white sturgeon could be considered a candidate for investigation into branchial \( \text{HCO}_3^-/\text{Cl}^- \) exchangers, such as those that have been hypothesized to be involved in \( \text{pHe} \) compensation during hypercarbia in, for example, the Atlantic stingray, \textit{D. sabina} (11, 46), as no \( \text{Cl}^-/\text{HCO}_3^- \) exchanger has yet been implicated in sturgeon.

It is important to note that the role and site of various acid-base transporters in the fish gill is still open to much debate, and no data exist for sturgeons. Therefore, many questions remain regarding the branchial mechanisms responsible for net acid excretion during environmental hypercarbia (17, 43). Furthermore, the role of the kidney in net acid excretion was not examined in this study. While most fishes studied to date are believed to excrete < 10% of net acid production via urine (43), this has not been confirmed in sturgeon.

Despite the relatively rapid \( \text{pHe} \) compensation in white sturgeon during exposure to 1.5 kPa \( \text{CO}_2 \), in white sturgeon exposed to 3 and 6 kPa \( \text{CO}_2 \), blood \( \text{pH} \) remained depressed for 48 h (Fig. 7, A and B). In a previous study (14), white sturgeon exposed to 4 kPa \( \text{CO}_2 \) also exhibited a blood acidosis and little \( \text{pHe} \) compensation (\( \text{pH} \) recovery of ~20% after 96 h). This is consistent with an apparent limitation to \( \text{pHe} \) compensation observed in other fishes (6, 25). However, during these exposures white sturgeon bicarbonate accumulation did not reach the proposed “bicarbonate concentration threshold” (i.e., 27–33 mmol/l), as net plasma [\( \text{HCO}_3^- \)] did not exceed 20 mmol/l at 3 or 6 kPa \( \text{CO}_2 \). The limit for net \( \text{HCO}_3^- \) accumu-
lation during hypercarbia in sturgeon may be lower than other fish; alternatively, this may be the result of the severe water acidification associated with hypercarbia of soft water (water pH \( \approx 5.5 \) and 4.5 at 3 and 6 kPa PCO\(_2\), respectively), as activity of branchial apically located V-ATPase has been shown to be inhibited at a water pH below pH of 5.5 in trout (34). There was no evidence of increased gill mucous production (visual inspection) or gill damage (as indicated by osmoregulatory status) in response to severe hypercarbia. Whatever the cause, clearly, in white sturgeon exposed to these higher CO\(_2\) tensions, pHe remained significantly depressed for the duration of experimental exposure (48 h).

pHi during hypercarbia exposure. In this study, white sturgeon RBC pHi exhibited a qualitatively similar pattern of change to whole blood pHi during hypercarbia exposure; as pHe recovered, so did RBC pHi (Figs. 1B; 8, A and B; 9). When RBC pHi values for all CO\(_2\) tensions and times were plotted against blood pHi, a significant positive correlation was observed [series 1 (all data), slope = 0.48, \( r^2 = 0.26 \), series 2 (all data), slope = 0.50, \( r^2 = 0.93 \)]. Many teleosts regulate RBC pHi during a plasma acidosis through the release of catacholamines and subsequent activation of RBC \( \beta\)-NHE (7). This acts to protect O\(_2\) uptake at the gills during a generalized acidosis in the presence of a Root effect, where oxygen-carrying capacity of the blood may be greatly reduced by a reduction in pH. As white sturgeon do not possess adrenergically-activated RBC \( \beta\)-NHE (4), the pHe-to-pHi relationship observed in this study might be expected a priori (7) and is consistent with that observed in the armoured catfish, which also lacks RBC \( \beta\)-NHE (8).

In the few studies where pHe and tissue pHi in fish exposed to hypercarbia have been studied simultaneously, changes in pHe were often reflected in the intracellular compartment (e.g., 57, 58). Consequently, if pHe recovery is limited, pHi would also presumably remain depressed and, due to the importance of pH to cellular processes, have severe consequences [e.g., decrease in myocardial contractile force (19)].

Fig. 7. Effect of exposure to normocarbia (air-equilibrated water) (○), or one of two hypercarbia treatments, 3 kPa (22.5 Torr) PCO\(_2\) (●) or 6 kPa (45 Torr) PCO\(_2\) (◇) on blood pHi and plasma [HCO\(_3^-\)] in cannulated white sturgeon over 48 h. Blood pHi is plotted as a function of time, sampled at 15 and 30 min, and 1, 3, 6, 12, 24, and 48 h (A), and against plasma [HCO\(_3^-\)] (B), represented on a pHi/HCO\(_3^-\)/CO\(_2\) plot. Values are presented as means ± SE. (n = 4 – 6). In A, letters indicate homogeneous subsets. In B, numbers on figure indicate time in hours, and dashed line indicates blood nonbicarbonate buffer line and is oriented through normocarbic data (normocarbic data presented in A, not shown in B for clarity).

Fig. 8. Effect of exposure to normocarbia (air-equilibrated water) (○) or one of two hypercarbia treatments, 3 kPa PCO\(_2\) (●) or 6 kPa PCO\(_2\) (◇), on RBC pHi as a function of time (A) or blood pHi (B) in cannulated white sturgeon over 48 h. In A, values are presented as means ± SE (only groups where n ≥ 3 are presented). Letters indicate time points within a treatment that is significantly different. In B, the correlation between blood pHi and RBC pHi was significant (slope = 0.52, \( r^2 = 0.90 \), \( P < 0.05 \)).
extracellular fluid comprised 25% and white muscle intracellular water the remaining 75% of total body water, and that pHi compensation can be accomplished within 6 h (as preliminary studies suggest), the rate of bicarbonate uptake from the environment at 6 kPa PCO2 over that first 6 h would be ~0.50 µmol HCO3/g water. This value is surprisingly similar to the rate found in P. pardalis (0.55 µmol HCO3/g water) calculated using a similar approach (8). While further investigation is necessary to verify this calculation, it is well within the capacity for net HCO3/Cl− exchange (8).

The relationship between pH and pHi in white sturgeon tissues described in this study differs from the commonly accepted pattern of pH recovery during a respiratory acidosis in fishes, i.e., the concurrent compensation of pH and tissue pHi (57, 58). Furthermore, this study suggests that, under certain conditions, pHi can be elevated above normocapnic levels (Figs. 1, B–C and 9) during a blood acidosis. Because pHi is closer to the equilibrium constant (pK) for the hydration of CO2, much smaller increases in [HCO3−] in the intracellular, relative to extracellular, space are required for pH recovery during a respiratory acidosis (Table 2). While preferentially regulating pHi over whole body pH may represent a reduced ionoregulatory cost at lower CO2 tensions, it is possibly the only feasible option for survival when PCO2 exceeds 2 kPa, the apparent limitation to pH compensation observed in fishes (6, 25).

While some protective responses have been observed in response to an acidosis in fish tissues and cells [e.g., down-regulation of protein synthesis (30)], few studies have demonstrated pHi protection during pHe acidosis in fishes during short-term (hours to days) exposure to hypercarbia. Currently, only the facultative air breather, P. pardalis, exhibits a similar pH regulatory response (8) of the magnitude seen in this study, although the marbled swamp eel, S. marmoratus, was able to protect heart and white muscle pH during the much less severe respiratory acidosis associated with hypoxia-induced air breathing (24). Evidence from examination of isolated tissue and cell preparations in other fish species suggests that considerable variability exist in the ability of tissues to regulate pHi during an induced acidosis (21, 29, 30, 49). For example, pattern in other fishes, white sturgeon completely protected pHi in heart, liver, brain, and white muscle during a respiratory acidosis induced by hypercarbia (Fig. 7A). This tissue pHi protection was not attributable to measured intrinsic buffering for heart, liver, and white muscle (Fig. 10), as measured values were quite low, even compared with the CO2-sensitive rainbow trout (40, 58). Although the technique used in this study may overestimate the buffer capacity by exposing titratable sites in vitro that may not be available in vivo (47, 51), actual values in sturgeon would be even lower than those reported here. Consequently, cellular transmembrane exchange of acid-base relevant ions must be responsible for pHi regulation in these tissues, which would ultimately elevate intracellular HCO3−.

Assuming that the net intracellular HCO3− uptake associated with pHi regulation represents uptake of HCO3− from the environmental water (presumably in exchange for Cl−) a simple calculation can provide insight into the rate of HCO3−/Cl− exchange that would be required. Assuming sturgeon

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**Fig. 9.** Relationship between blood extracellular pH (pHe) and pHi of RBC (●), white muscle (●) and liver (○) (A) and heart (●) and brain (○) (B) of white sturgeon following 48 h of exposure to either normocarbia (air-equilibrated water) or hypercarbia (3 and 6 kPa PCO2). Tissues are presented in separate panels for clarity. Values are presented as means ± SE (n = 4–6). Correlations between raw data for pHe and tissue pHi are described by the following lines: RBC: m = 0.48, r² = 0.96, P < 0.05; heart: slope = −0.14, r² = 0.67, P < 0.05; brain: slope = −0.24, r² = 0.72, P < 0.05; liver: not significant; white muscle: not significant. Mean values of pHe and RBC pHi were significantly different between treatments; mean pHi values of other tissues were not different between treatments (not indicated for clarity).

**Fig. 10.** Relationship between blood pH and plasma [HCO3−] in blood equilibrated in vitro at 0.5, 1, 2, 4, 6, and 10 kPa (3.75, 7.5, 15, 30, 45, and 75 Torr) PCO2. Values are means ± SE (n = 4). Nonbicarbonate buffer capacity of blood (βBH = 11.9 mmol HCO3−·L−1·pH unit−1, r² = 0.878) was calculated from the slope of the best-fit linear regression over in vivo pHi values.
rainbow trout hepatocytes have both NHE and Na\(^+\)/HCO\(_3\)\(^-\) cotransporters that contribute to pHi regulation during acid loading (18). In addition, NHE activity is higher at low O\(_2\) levels, suggesting a role in correcting for anoxia-induced acidosis (54). On the other hand, in hepatocytes isolated from goldfish (Carassius auratus), a far more anoxia-tolerant fish, a sodium-independent Cl\(^-\)/HCO\(_3\)\(^-\) exchanger was experimentally determined to increase acid excretion during chemical anoxia, implying that this pH regulatory mechanism may be contributing to hypoxia tolerance (29). Under conditions of hypercarbia, when plasma HCO\(_3\)\(^-\) levels are elevated two- to tenfold over normal levels, Cl\(^-\)/HCO\(_3\)\(^-\) exchange may be more favored energetically; these energetic savings could be associated with observations of overcompensatory pHi response in sturgeon tissues exposed to elevated CO\(_2\). Thus, a Cl\(^-\)/HCO\(_3\)\(^-\) exchanger remains for us a strong candidate for cellular pH protection in white sturgeon during hypercarbia. Identification and characterization of the specific mechanisms involved in the remarkable capacity of tissue pHi regulation in white sturgeon remains an exciting research area for further experimental investigation.

**Perspectives and Significance**

Clearly, white sturgeon have the capacity to alter net epithelial acid-base relevant ion transport to drive pHe recovery during moderate levels of hypercarbia (1.5 kPa), despite previous evidence to the contrary (14). Furthermore, regardless of the severity of the extracellular acidosis induced (>0.6 pH units), white sturgeon were able to regulate pHi in heart, white muscle, brain, and liver at normocarbic levels during a transient (6 h) or prolonged (48 h) extracellular acidosis. White sturgeon are currently the most basal fish to exhibit this pattern of pHi protection, as it does not occur in the osmoconforming hagfishes (6) or elasmobranchs (26, 58). Both of the latter groups have much higher plasma [Cl\(^-\)] than osmoregulating fishes, which may be the basis for a higher “bicarbonate threshold” permitting the use of branchial Cl\(^-\)/HCO\(_3\)\(^-\) exchange to compensate for the acidosis induced by much higher Pco\(_2\) levels than in other fishes investigated. This certainly appears to be the case in hagfish (6), and high CO\(_2\) tolerance has been observed in some elasmobranchs in the absence of pHi protection (23). As sturgeon represent the most basal osmo/ionoregulating fish examined to date, robust pHi regulation in critical tissues (i.e., heart) may have arisen as a means of protecting these organs from acid-loading events that could no longer be compensated for extracellularly through net branchial Cl\(^-\)/HCO\(_3\)\(^-\) exchange. Further exploration of this premise is needed and could begin with investigation into other more basal osmoregulating fish.

To date, few fish species have exhibited the hypercarbia tolerance observed in this study (6). While CO\(_2\) tolerance in fish may be more common than previously thought (6), especially in tropical fresh-water systems, which may experience diurnal periods of hypercarbia (55), it is unclear why sturgeon, limited to the northern hemisphere and mostly temperate ecosystems, would have evolved such tolerance. On the other hand, the sturgeon lineage departed from the teleost ancestry 250–300 million years ago. Since this time, global and local conditions have often created extensive tropical aquatic systems that may have been similar to those currently observed in the Amazon Basin, where contributing factors, such as thick surface vegetation, poor water mixing, high flora or fauna biomass, and anaerobic metabolism of microorganisms can lead to Pwco\(_2\) levels as great as 8 kPa, or a 20- to 30-fold increase over the resting arterial Paco\(_2\) of fish (8, 24, 55). Consequently, sturgeon may have been historically exposed to these very hypercarbic conditions, which could not have been compensated for by branchial HCO\(_3\)\(^-\)/Cl\(^-\) exchange alone. Whether increased capacity for tissue pHi regulation represents a response common to all more-derived hypercarbia-tolerant fish [such as eel (37)] is unknown (6), but this study clearly supports additional investigation into that hypothesis.
This finding of complete tissue pHi compensation is the first for a non-air-breathing fish, as the other two fish species exhibiting tissue pHi protection during elevated PaCO₂ are the more derived facultative air breathers, S. marmoratus (24) and P. pardalis (8). Preliminary evidence suggests that the facultative air-breather, Amia calva (also more derived than sturgeon) may exhibit this response as well (Baker DW and Brauner CJ, personal observation). Therefore, our findings support the hypothesis that the capacity to regulate pHi during pHex depression and the resultant increase in CO₂ tolerance may have been an exaptation (i.e., preadaptation) for air breathing in facultative air breathers where a respiratory acidosis is incurred (6). While this remains to be validated (see Ref. 6), such a conclusion would reflect on Ultsch’s (55) proposal that freshwater hypercarbia has been overlooked as a parameter influencing the transition of life from water to land. Clearly, a number of exciting avenues for investigation of pHi regulation during hypercarbia await further address.

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