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# Metabolic changes associated with acid–base regulation during hypercarbia in the $CO_2$-tolerant chondrostean, white sturgeon (*Acipenser transmontanus*)

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ABSTRACT

$CO_2$ tolerance in white sturgeon is associated with the ability to tightly regulate intracellular pH (pHi) despite a large reduction in extracellular pH (pHe) termed preferential pHi regulation. How this regulatory response affects whole animal metabolic rate is unknown. Accordingly, we characterized oxygen consumption rate ($\dot{M}_{O_2}$) and metabolically-relevant organismal and cellular responses in white sturgeon during exposure to hypercarbia. White sturgeon were able to protect intracellular pH (pHi) in liver and white muscle as early as 6 h (the earliest time period investigated) following exposure to severe (sub-lethal) hypercarbia (45 and 90 mmHg $PCO_2$). Sturgeon exposed to 15 and 30 mmHg $PCO_2$ exhibited pHe compensation and significant increases in $\dot{M}_{O_2}$ (up to 80% greater than control values). In contrast, severe hypercarbia (≥45 mmHg $PCO_2$) elicited an uncompensated reduction in pHe (up to ~1.0 pH units) and red blood cells (as great as ~0.5 pH units), and was accompanied by 30 and 60% reductions in $\dot{M}_{O_2}$, respectively. While behavioral, respiratory and cellular responses to hypercarbia were observed, none corresponded well with the pattern or magnitude of changes in $\dot{M}_{O_2}$. The findings of this research provide empirical support for the hypothesis that preferential pHi regulation is not metabolically costly, and thus may have been a strategy strongly selected for in fishes encountering short-term hypercarbia.



## 1. Introduction

Aquatic hypercarbia (elevated water $CO_2$) can occur in highly productive estuaries, tide pools, and seasonally isolated fresh water ponds, in some cases resulting in a large and rapid elevation of water $PCO_2$, (>60 mmHg, Ultsch, 1996; Heisler et al., 1982). In fish, aquatic hypercarbia is typically associated with behavioral (*e.g.*, avoidance), respiratory (*e.g.*, increased ventilatory rates, Milsom, 2002), physiological (*e.g.*, increased net acid excretion, Heisler, 1999) and morphological (*e.g.*, increased surface area of chloride cells, Perry and Gilmour, 2006) responses, and commonly includes a well-described extracellular pH (pHe) compensatory response, mainly through branchial mechanisms (90%, Deigweiher et al., 2008) to correct the induced respiratory acidosis. Exposure to more severe hypercarbia (>15 mmHg $PCO_2$) induces an intra- and extracellular acidosis beyond the capacity for pHe compensation in most fishes (Heisler, 1986; Brauner and Baker, 2009), and the prolonged acidosis likely contributes to the rapid morbidity observed (*e.g.*, *Pagrus major*, *Paralichthys olivaceus*, *Seriola quinqueradiata*, and *Mustelus manazo*, Hayashi et al., 2004; Ishimatsu et al., 2005). The basis for this limit to pHe compensation during exposure to severe hypercarbia remains controversial, but may be related to environmental, extracellular and intracellular ionic composition (*e.g.*, environmental and extracellular [$HCO_3^-$], Heisler, 1986; environmental and intracellular pH, Lin and Randall, 1993; Parks et al., 2008; environmental [$Ca^{2+}$], Heisler, 1999; intracellular and environmental [$Na^+$], Parks et al., 2008; and extracellular [$Cl^-$], Brauner and Baker, 2009).

A number of fishes have demonstrated superior $CO_2$ tolerance far beyond this apparent limit to pHe compensation (*i.e.*, ≫15 mmHg $PCO_2$) which appears to be associated with complete intracellular pH (pHi) regulation (in heart, liver, brain and white muscle) in the face of a large sustained reduction in pHe, termed preferential pHi regulation (*Synbranchus marmoratus*, Heisler, 1982; armored catfish, *Pterygoplichthys pardalis*, Brauner et al., 2004; and white sturgeon, *Acipenser transmontanus*; Baker et al., 2009a). The cellular mechanisms responsible for preferential pHi regulation remain to be elucidated, and while the ubiquity of preferential pHi regulation is also unknown, few fishes investigated to date exhibit this capacity. Whether there is a metabolic cost associated with preferential pHi regulation is not known and is the focus of this study.

Preferential pHi regulation is dependent on net acid extrusion which may require ATP, directly (for example, for V-type $H^+$-ATPase) or indirectly [such as in the removal of $Na^+$ accumulated *via* $Na^+/H^+$ exchange through increased $Na^+/K^+$ ATPase (NKA) activity]. This ATP demand could be fuelled either aerobically, and be associated with an increase in organismal $\dot{M}_{O_2}$, or anaerobically, and be associated with lactate production. Alternatively, preferential pHi regulation may be metabolically less costly than pHe compensation, as suggested by previous calculations of net acid equivalents necessary to drive pHi recovery (Brauner et al., 2004; Brauner and Baker, 2009). Thus, changes

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in $\dot{M}_{O_2}$ and tissue lactate concentrations during hypercarbia may provide insight into metabolic costs associated with pH compensation.

However, attributing changes in $\dot{M}_{O_2}$ during aquatic hypercarbia exclusively to acid–base regulatory response would be remiss, as they could also be the result of organismal or cellular responses not directly related to pH compensation. For example, aversion or escape behavior (*e.g.*, increased swimming activity) and ventilation frequency can affect metabolic rates. As well, metabolic rate can be reduced to survive challenging conditions, a strategy commonly referred to as metabolic suppression (Hochachka and Somero, 2002), and this is commonly accomplished by rapidly and severely reducing activity of expensive cellular processes, such as NKA and protein turnover in less critical tissues (>80% within 12–24 h in goldfish liver, Jibb and Richards, 2008). Thus, these factors must also be taken into account when interpreting the cost of acid–base regulation based upon changes in $\dot{M}_{O_2}$.

We hypothesize that preferential pHi regulation does not require substantial increases in metabolic demand, and thus may occur in the absence of increases in metabolic rate. White sturgeon, *Acipenser transmontanus*, are particularly well suited to address this question, as they not only exhibit pHe compensation at low $CO_2$ tensions as do most fishes, but also preferential pHi regulation at higher $CO_2$ tensions despite sustained reductions in pHe of almost a full pH unit (Baker et al., 2009a). To test this hypothesis, here we investigated pH compensation (both pHe and intracellular) during exposure to a range of $CO_2$ levels, and concurrently measured a suite of metabolically-relevant parameters at the whole animal and cellular levels to provide insight into metabolic adjustments during hypercarbia. More specifically, in response to short term hypercarbia (up to 48 h of exposure to up to $\leq$90 mmHg $PCO_2$), we characterized pHe and pHi compensation, rates of oxygen consumption (*i.e.*, $\dot{M}_{O_2}$), and metabolically relevant organismal (*e.g.*, spontaneous activity, ventilation frequency) and biochemical (*i.e.*, NKA activity and maximal rate of protein synthesis in the liver, and lactate levels in heart and white muscle) parameters.

## 2. Materials and methods

### 2.1. Animals and rearing conditions

Hatchery-reared, juvenile white sturgeon, *A. transmontanus*, (1+, 10 to 30 g) progeny of wild stock, were provided by the Upper Columbia White Sturgeon Recovery Initiative's white sturgeon hatchery in Wardner, B.C. All animals were held at the Department of Zoology, University of British Columbia (UBC), Vancouver, B.C., in large, aerated outdoor flow-through tanks ($O_2$>90% saturation, $CO_2$<0.2 mmHg, T~10–12 °C, fish density<20 kg $m^{-3}$ water) in Vancouver dechlorinated city water (water hardness: <5 mg $L^{-1}$ [$CaCO_3$], alkalinity: 3–4 mg $L^{-1}$ [$CaCO_3$], pH: 6.7–7.0, [$Na^+$] and [$Cl^-$]<2 mg $L^{-1}$). Fish were fed a commercial diet to satiation daily, but food was withheld 24 h prior to experimentation. Mortality rate was less than 0.5% $week^{-1}$ over the 3 month holding and experimental period. All protocols complied with the guidelines approved by the Canadian Council on Animal Care, UBC ACC protocol # A07-0080.

### 2.2. Experimental design

In all experiments, system design constraints limited investigation to only one $CO_2$ tension at a time, but the $CO_2$ tension for a given trial was chosen at random. In each case, all experimental boxes were plumbed into the same $CO_2$ equilibrated recirculating system, thereby ensuring identical $CO_2$ exposures to all animals within each treatment. In all experiments, $CO_2$ tensions were verified at the beginning and end of the experiment, and every 8 h in those lasting longer than 6 h using a Radiometer $PCO_2$ electrode (E5036) displayed on a Radiometer PHM 71.

#### 2.2.1. Series 1: the effect of hypercarbia on hematology and acid–base physiology

White sturgeon (~50–150 g) were transferred in water into darkened boxes (30 L each, 10 fish per box) plumbed into a thermostated re-circulating (flow rate ~3 L $min^{-1}$) system, and pre-equilibrated to one of three $CO_2$ tensions (ambient, 45, and 90 mmHg $PCO_2$). Water $O_2$ levels remained above 85% air saturated values during all exposures. Target $CO_2$ tensions were achieved by aerating a mixing tank plumbed into the re-circulating system with preset rates of air and 100% $CO_2$ using a Cameron Gas Mixer. After 6 h, white sturgeon were transferred to water containing MS-222 (0.3 g $L^{-1}$, buffered with $NaHCO_3$) equilibrated with the experimental $CO_2$ tension. After ventilation ceased (<1 min), blood (1 mL) was immediately drawn from the caudal vein *via* a sterile lithium-heparin-rinsed (150 i.u. $mL^{-1}$ heparin) syringe (10 mL syringe, 23 G needle), and placed on ice. Fish were subsequently killed *via* spinalectomy, and sections of liver and white muscle were excised and flash frozen in $LN_2$ for later measurements of pHi. Blood was then separated into two aliquots, and hematological parameters {*i.e.*, pH, hematocrit (HCT), and hemoglobin concentration [Hb]} and plasma total $CO_2$ ($TCO_2$) ($CO_2$ Analyzer; model 965, Corning) and plasma [$Cl^-$] (HBI model 4425000; digital chloridometer) were measured using previously described methods (Brauner et al., 2004; Baker et al., 2009b) and solubility and dissociation constants (Boutilier et al., 1984). Separated RBC pellets were analyzed for pHi using the freeze–thaw method (Zeidler and Kim, 1977), while tissue pHi was measured using the metabolic inhibitor tissue homogenate method of Pörtner and colleagues (1990), recently validated for tissues exposed to high $PCO_2$s (Baker et al., 2009b).

#### 2.2.2. Series 2: the effect of hypercarbia on $\dot{M}_{O_2}$

Juvenile white sturgeon (70–120 g, n = 8 for each $CO_2$ tensions, except 60 mmHg $PCO_2$ where n = 4) were transferred without air exposure to one chamber (2.4 L) of a 4-chamber, intermittent-flow respirometry system (Loligo Systems, Hobro, Denmark) supplied with aerated water at 1 L $min^{-1}$. Fish were allowed 24 h to acclimate prior to $\dot{M}_{O_2}$ measurements, which preliminary experiments indicated was sufficient time for $\dot{M}_{O_2}$ to stabilize. Chambers were submerged in a water bath to ensure a constant temperature (10 ± 0.5 °C) during the 6-week experimental period. A 5:15 min flush-to-measurement cycle was used and oxygen content of the water was measured every second during the 15 min recirculation cycle using a MINI-DO probe (Loligo Systems), which had been calibrated with anoxic and 100% air saturated water prior to the introduction of each fish (as per Eliason et al., 2008). Each chamber was fitted with a recirculation pump to ensure adequate water mixing during the measurement cycle.

At the beginning of the experiment, the water source for the flush cycle either remained at ambient levels of $CO_2$ or was switched to one of the following pre-equilibrated $CO_2$ tensions: 15, 30, 45 or 60 mmHg $PCO_2$. $\dot{M}_{O_2}$ was recorded using LoliResp4 software (Loligo Systems). Each experiment lasted 48 h during which $\dot{M}_{O_2}$ was continuously measured (with the limitations of the flush:measurement cycle described above). Following the 48-h exposure period, fish were transferred in water to a recovery tank separate from stock fish.

#### 2.2.3. Series 3: the effect of hypercarbia on organismal, physiological and cellular energetically-relevant parameters

In this series, organismal [tail beat ($f_T$) and ventilation frequency ($f_V$)], physiological (pHe and plasma bicarbonate concentration) and biochemical parameters (liver cell-free maximal rate of protein synthesis, liver maximal NKA activity, and heart and white muscle lactate concentration) were measured. White sturgeon were individually transferred without air exposure to one of five darkened boxes (10 fish per 30 L box) all previously equilibrated to one of ambient, 15, 30, 45, or 60 mmHg $PCO_2$. Fish were monitored from directly above for 1 min using video media (Sony DCR DVD 650) every 10 min for the first 3 h of $CO_2$ exposure and then for 1 min prior to terminal

sampling at each of 3, 6, 12, 24 and 48 h time points following initial $CO_2$ exposure. Tail beat frequency ($f_T$) and ventilation frequency ($f_V$) of white sturgeon were later analyzed from these recordings. Fish were then euthanized as described in Series 1. Blood was obtained and blood pH and plasma $TCO_2$ were measured as described in Series 1. Liver, white muscle and heart were excised within 1 min of euthanasia and flash frozen and stored in liquid nitrogen. Liver tissue was later analyzed for a) maximal relative activity of NKA, an indicator of potential NKA activity and b) cell-free protein translation rate, an indicator of capacity for protein synthesis, following 12 h of hypercarbia. Heart and white muscle were analyzed for tissue lactate concentration following 24 h of hypercarbia.

White sturgeon $f_T$ ($min^{-1}$), a quantitative proxy for qualitative changes in activity levels, was measured as the number of tail beats over a 30-s period when fish were continuously in view. $f_V$ ($min^{-1}$) was quantified as the number of opercular contractions over a 30-s period. Quantification of ventilation amplitude was abandoned after determining that changes associated with all treatments were below the detectable limits of the video resolution. All quantification was performed by a single observer, with verification of randomly selected periods of the video recording by a second observer.

An NADH-linked assay and spectrometry were used to measure liver NKA activity as described previously (Else and Wu, 1999; modified as in Bystriansky et al., 2006). Briefly, liver tissue (~80 mg) was sonicated (Kontes) on ice in SEI buffer (pH = 7.5; 150 mmol $L^{-1}$ sucrose, 10 mmol $L^{-1}$ EDTA, 50 mmol $L^{-1}$ imidazole) and centrifuged (1 min @ 5000 *g*) at 4 °C. Protein content of tissue homogenates was measured using the Bio-Rad standard protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was assayed (VersaMax Microplate Reader, Molecular Devices) for ATPase activity in triplicate in the presence and absence of the NKA-specific inhibitor ouabain (final concentration 1 mmol $L^{-1}$), and the difference in the rate of NADH oxidation between the two conditions was used to calculate NKA activity.

Liver protein synthesis rates were determined following previously described methods (Rider et al., 2006; Jibb and Richards, 2008). Frozen liver was homogenized at 1:5 (w/v) in ice-cold extraction buffer containing 50 mmol $L^{-1}$ Hepes (pH 7.4), 250 mmol $L^{-1}$ sucrose, 20 mmol $L^{-1}$ NaF; 5 mmol $L^{-1}$ sodium pyrophosphate; 1 mmol $L^{-1}$ EDTA, and 1 mmol $L^{-1}$ EGTA, and then centrifuged at 14,000 *g* for 15 min at 4 °C. The resulting supernatant was removed and stored at −80 °C. Sephadex G-25 columns (GE Healthcare, Piscataway, NJ, USA) were equilibrated with an intracellular buffer containing 50 mmol $L^{-1}$ Hepes (pH 7.4), 200 mmol $L^{-1}$ potassium acetate, 5 mmol $L^{-1}$ magnesium acetate, 1 mmol $L^{-1}$ DTT, 5 g $mL^{-1}$ leupeptin, 1 mmol $L^{-1}$ benzamidine, and 1 mmol $L^{-1}$ PMSF as instructed by the column manufacturer, and thawed tissue extracts (0.5 mL) were filtered through these columns to remove endogenous amino acids. Protein content of this filtrate was measured as above. To determine protein synthesis rates, a 50 μL aliquot of the filtrate was added to assay buffer containing 50 μg $mL^{-1}$ total RNA prepared from sturgeon liver using the Tri-Reagent (Sigma Aldrich Chemical Co.) method (described in detail in Scott et al., 2005), and 20 mmol $L^{-1}$ of each amino acid (except leucine) to a final volume of 100 μL. The reaction was started by adding 0.9 μL of 20 μmol activated leucine stock containing L-[4,5-$^3$H]-leucine (~300 cpm $pmol^{-1}$) and incubated at 25 °C for 90 min. Following incubation, the reaction was stopped with the addition of 1 mL 10% trichloroacetic acid. Smaple were then placed on ice (10 min), and centrifuged at 10,000 *g* for 5 min to collect precipitated proteins. The pellet was resuspended in 0.2 mL of 0.1 mol $L^{-1}$ sodium hydroxide and re-precipitated in 1 mL of 5% trichloroacetic acid. After a further 10 min on ice, proteins were collected by centrifugation and subjected to an additional wash. Following this wash, proteins were solubilized in 1 mL formic acid and 0.9 mL of the solubilized protein solution was taken for counting in 10 mL of toluene-based scintillate on a LS 1801 liquid scintillation counter (Beckman Coulter, Mississauga, ON, Canada). Cellular protein synthesis rate is expressed as pmol leucine mg total $protein^{-1}$ $h^{-1}$.

For determination of tissue [lactate], ~20 mg Lyophilized white muscle or heart was homogenized in ice-cold 8% perchloric acid for 15 s using a sonicator (Kontes). Homogenates were then centrifuged at 20,000 *g* for 5 min at 4 °C and the supernatant adjusted to ~pH 7.6 with potassium carbonate. Neutralized extracts were centrifuged (5 min at 20,000 *g*; 4 °C) and the supernatant was immediately frozen in $LN_2$ and stored at −80 °C until use. These extracts were then used for enzymatic determination of tissue [lactate] *via* the method described by Bergmeyer (1983).

### 2.3. Calculations and statistical analyses

All values are presented as mean ± s.e.m., with sample sizes indicated in text. Whole animal $\dot{M}_{O_2}$ over time (Series 2) was analyzed using a two-way RM ANOVA, with $CO_2$ and time as factors. In all other two-factor experiments, a two-way ANOVA (factors: $CO_2$ × time) was used to analyze the effects of $CO_2$ tension and time. Where a significant interaction was detected, one-way RM ANOVA or one-way ANOVA as appropriate was used to detect effects within each factor. When differences were indicated, Student–Newman–Keuls or Dunnett's post-hoc tests (as appropriate) were used to determine homogeneous subsets. When assumptions were violated, an ANOVA on ranks was used. The effect of $CO_2$ on parameters determined at a single time point (such as those obtained following terminal sampling: hematology, tissue pHi, lactate, protein synthesis rate, and NKA activity) or mean $\dot{M}_{O_2}$ (*i.e.*, over 48 h) were analyzed using one-way ANOVA and SNK or Dunnett's (with control values from fish exposed to ambient $CO_2$ tension) post-hoc tests. In all cases, α of 5% ($p = 0.05$) was selected to signify statistically significant differences. All statistical tests were performed with SigmaSTAT (version 10.0).

## 3. Results

### 3.1. Series 1: the effect of hypercarbia on hematology and acid–base physiology

White sturgeon exposed to 45 or 90 mmHg $PCO_2$ for 6 h exhibited a significant decrease in pHe, RBC pHi (Fig. 1), and plasma [$Cl^-$] (Table 1) and a significant increase in plasma [$HCO_3^-$] (Table 1) relative to control values. A significant increase in liver and white muscle pHi occurred following 6 h of exposure to 45 (liver and white muscle) or 90 (liver only) mmHg $PCO_2$ (Fig. 1). White sturgeon HCT, [Hb], and

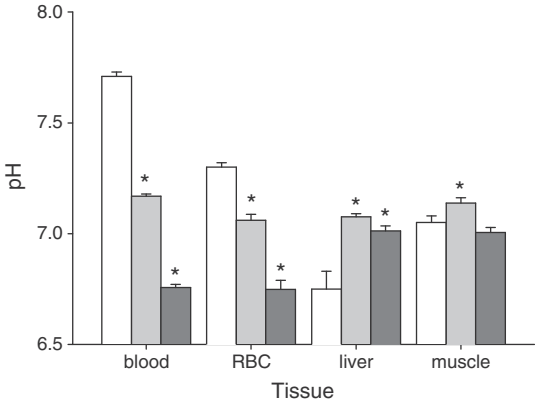


**Fig. 1.** The effect of 6 h of hypercarbia (normocarbia, white bars; 45 mmHg $PCO_2$, light bars; 90 mmHg $PCO_2$ dark bars) on white sturgeon blood pH (pHe) or intracellular pH (pHi) of red blood cells (RBC), liver, and white muscle. Values are means ± s.e.m. ($n = 10$). An asterisk indicates a statistically significant difference from the normocarbia exposed group.

**Table 1**
The effect of short-term (6 h) hypercarbia (45 and 90 mmHg $PCO_2$) on hematocrit (HCT,%), hemoglobin concentration ([Hb]; mmol $L^{-1}$), mean cell hemoglobin concentration (MCHC), plasma bicarbonate concentration (mmol $L^{-1}$), and plasma chloride concentration (mmol $L^{-1}$) in white sturgeon.

| $PCO_2$ (mmHg) | HCT (%) | [Hb] (mmol $L^{-1}$) | MCHC (mmol Hb $L^{-1}$ packed RBCs) | $[HCO_3^-]$ (mmol $L^{-1}$) | $[Cl^-]$ (mmol $L^{-1}$) |
|---|---|---|---|---|---|
| Normocarbia | 30.8 ± 2.5 | 0.85 ± 0.06 | 2.81 ± 0.23 | 6.8 ± 1.1 | 119.3 ± 2.9 |
| 45 | 32.5 ± 2.7 | 0.77 ± 0.09 | 2.30 ± 0.16 | 18.5 ± 0.7* | 94.6 ± 3.03* |
| 90 | 24.0 ± 1.5* | 0.51 ± 0.03* | 2.19 ± 0.16* | 10.3 ± 0.7* | 88.1 ± 1.7* |

Values are mean ± s.e.m. An asterisk indicates a significant difference from the control treatment.

MCHC were significantly lower at 90, but not 45, mmHg $PCO_2$ (Table 1) relative to control values.

### 3.2. Series 2: the effect of hypercarbia on $\dot{M}_{O_2}$

In white sturgeon exposed to hypercarbia, mean $\dot{M}_{O_2}$ values calculated over the entire 48 h exposure duration were significantly higher at 30 mmHg $PCO_2$ and significantly lower at both 45 and 60 mmHg $PCO_2$ than in those exposed to both ambient and 15 mmHg $PCO_2$ (one-way ANOVA, $p<0.01$; Fig. 2A). Fig. 2B summarizes the temporal effects of $CO_2$ exposure on $\dot{M}_{O_2}$ (two-way RM ANOVA, interaction term, $p<0.001$). $\dot{M}_{O_2}$ of white sturgeon exposed to 15 mmHg $PCO_2$ was elevated between 36 h and 42 h, but did not differ significantly from ambient levels at any other time point (Fig. 3B). Fish exposed to 30 mmHg $PCO_2$ exhibited increased $\dot{M}_{O_2}$ relative to control fish in both the first 6 h, and between 24 h and 48 h (Fig. 3B). Fish exposed to 45 and 60 mmHg $PCO_2$ exhibited a significant reduction in $\dot{M}_{O_2}$ relative to ambient $PCO_2$ exposed fish throughout the duration of the exposure (Fig. 3B), with these changes occurring as early as within 90 min of transfer (data not shown).

### 3.3. Series 3: the effect of hypercarbia on organismal, physiological and cellular energetically-relevant parameters

There was an overall effect of $CO_2$ treatment and time on $f_T$, but no significant interaction (two-way ANOVA, Fig. 3). All $CO_2$-exposed fishes exhibited a significantly reduced $f_T$ when compared to fish held at ambient $CO_2$ levels, which matched qualitative observations of reduced activity levels. Also, $f_T$ of sturgeon exposed to 45 and 60 mmHg $PCO_2$ were significantly lower than in those exposed to 15 mmHg $PCO_2$. In fish exposed to $CO_2$, $f_T$ was similar to 3 h values within each treatment by 30 min (data not shown). $f_T$ was very low ($<4\ min^{-1}$) at all sampling periods over 48 h at 45 and 60 mmHg $PCO_2$ (Fig. 3), but even in control fish $f_T$ was low ($\sim 30\ min^{-1}$) indicative of low levels of spontaneous activity. There was also an overall effect of $CO_2$ exposure and time on $f_V$, but no significant interaction (Fig. 4). White sturgeon $f_V$ was significantly different between all treatments, but with respect to controls, $f_V$ was significantly elevated at 15 and 30 mmHg $PCO_2$ and significantly reduced at 45 and 60 mmHg $PCO_2$ (Fig. 4). Within each $CO_2$ treatment, changes in $f_V$ were rapidly induced (*i.e.*, within 10 min, data not shown).

In all groups exposed to elevated $CO_2$, pHe decreased and plasma $[HCO_3^-]$ increased by 3 h relative to control values (Fig. 5A, B). In fish exposed to 15 and 30 mmHg $PCO_2$, pHe was significantly higher at both 24 h and 48 h compared to the lowest measured value (at 6 h and 12 h respectively) within that $CO_2$ exposure (Fig. 5A, as indicated by an asterisk). Also, plasma $[HCO_3^-]$ was significantly higher in fish exposed to 15 and 30 mmHg $PCO_2$ at 12 h, 24 h and 48 h or 24 h

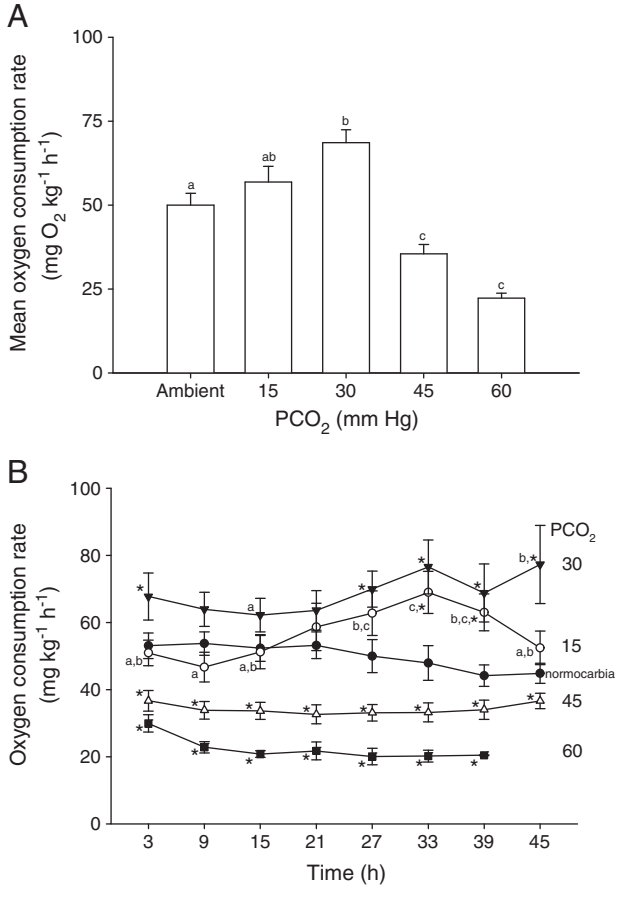


**Fig. 2.** The effect of 48 h of hypercarbia on A) overall mean oxygen consumption rate ($\dot{M}_{O_2}$) and B) $\dot{M}_{O_2}$ over time (binned in 6 h periods) of white sturgeon (normocarbia, filled circles; 15 mmHg $PCO_2$, open circles; 30 mmHg $PCO_2$, filled inverted triangles; 45 mmHg $PCO_2$, open triangles; and 60 mmHg $PCO_2$, filled squares). Values are means ± s.e.m. (n = 8 for each treatment, except 60 mmHg $PCO_2$ where n = 4). An asterisk indicates a significant difference from normocarbic treatment at a given sampling time. Letters that differ indicate significant differences within a treatment. At 60 mmHg, only three fish survived 36 h, and none survived past 45 h.

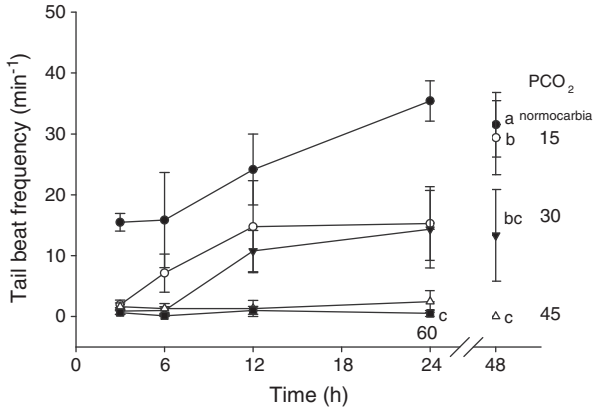


**Fig. 3.** The effect of 48 h of hypercarbia on tail beat frequency ($f_T$) of white sturgeon (normocarbia, filled circles; 15 mmHg $PCO_2$, open circles; 30 mmHg $PCO_2$, filled inverted triangles; 45 mmHg $PCO_2$, open triangles; and 60 mmHg $PCO_2$, filled squares). Values are means ± s.e.m. (n = 6–10 for each treatment, as limited by video analysis). There is an overall effect of $CO_2$ treatment and time, but no significant interaction. Letters that differ indicate differences between main treatment effects of $CO_2$. At 60 mmHg, no fish survived 48 h.

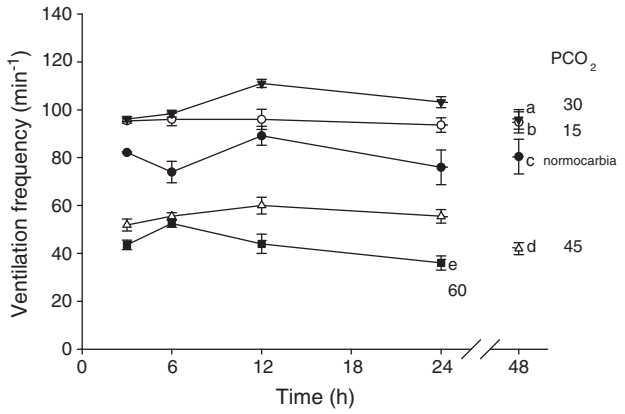


**Fig. 4.** The effect of 48 h of hypercarbia on ventilation frequency ($f_V$) of white sturgeon (normocarbia, filled circles; 15 mmHg $PCO_2$, open circles; 30 mmHg $PCO_2$, filled inverted triangles; 45 mmHg $PCO_2$, open triangles; and 60 mmHg $PCO_2$, filled squares). Values are means ± s.e.m. (n = 4–8 for each value, as limited by video analysis). There is an overall effect of $CO_2$ treatment and time, but no significant interaction. Letters that differ indicate differences between main treatment effects of $CO_2$. At 60 mmHg, no fish survived 48 h.

and 48 h, respectively, relative to 3 h values (Fig. 5B, as indicated by an asterisk). In fish exposed to 45 and 60 mmHg $PCO_2$, no significant within-treatment difference was detected between pHe and $HCO_3^-$ values at 3 h relative to values obtained at subsequent sampling times.

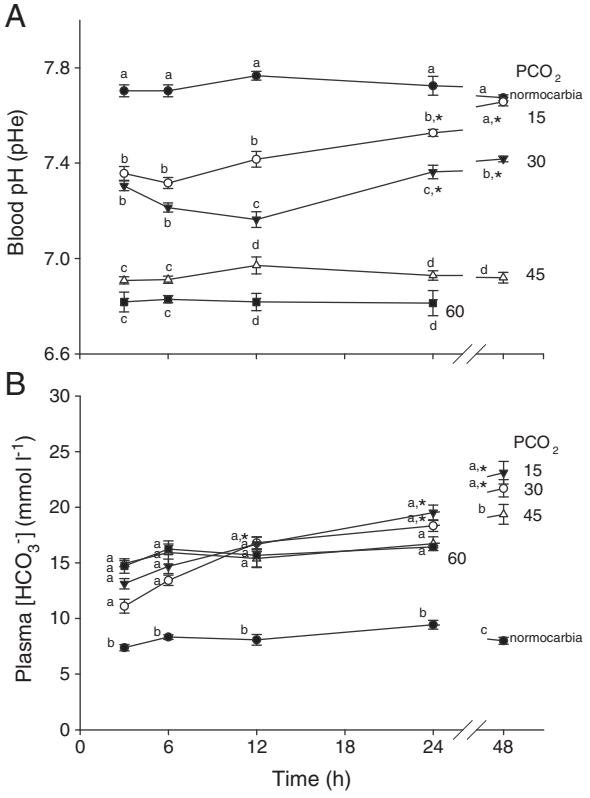


**Fig. 5.** The effect of 48 h of hypercarbia on A) whole blood pH and B) plasma [$HCO_3^-$] of white sturgeon (normocarbia, filled circles; 15 mmHg $PCO_2$, open circles; 30 mmHg $PCO_2$, filled inverted triangles; 45 mmHg $PCO_2$, open triangles; and 60 mmHg $PCO_2$, filled squares). Values represent mean ± s.e.m. (n = 7 per group, except 60 mmHg $PCO_2$ where n = 4). Letters that differ indicate significant differences between treatments at a given sample time. An asterisk indicates a significant increase over lowest measured value within a treatment (see text for details). At 60 mmHg, no fish survived 48 h.

Liver cell-free protein synthesis rate (as indicated by radioactive leucine incorporated into proteins produced from liver homogenates) was unaffected by 12 h exposure to any level of hypercarbia (overall, 1.59 ± 0.07 pmol leucine mg total protein$^{-1}$ h$^{-1}$). In contrast, maximal liver NKA activity was significantly lower following 24 h of exposure to all $CO_2$ levels (on average, 60% lower; Fig. 6). Tissue lactate concentration following 24 h was significantly higher in heart tissue at 30 mmHg $PCO_2$ but significantly lower in white muscle at 45 mmHg $PCO_2$ compared to control values (Fig. 7).

## 4. Discussion

This study provides additional evidence for rapidly-activated pHi regulation, as the large uncompensated acidosis in blood and RBC at 45 and 90 mmHg $PCO_2$ was not accompanied by acidification in liver or muscle tissues. Relative to normocarbic rates, $\dot{M}_{O_2}$ increased during exposure to 15 and 30 mmHg $PCO_2$ during which time complete pHe recovery was observed (associated with a net $HCO_3^-$ accumulation in exchange for $Cl^-$). However $\dot{M}_{O_2}$ decreased at 45 and 60 mmHg $PCO_2$ during which time pHe compensation was negligible but complete preferential pHi regulation was observed. This pattern of an increase in $\dot{M}_{O_2}$ at low $CO_2$ tensions and a decrease at high $CO_2$ tensions was qualitatively matched by changes in ventilation frequency ($f_V$). In contrast, behavioral ($f_T$) and cellular (lactate accumulation, NKA activity, and rates of protein synthesis) responses did not correspond well to the pattern or magnitude of changes in $\dot{M}_{O_2}$. Thus, preferential pHi regulation in white sturgeon was not associated with an increase in overall metabolic costs. In our assessment of metabolically-relevant parameters, only the presence of pHe compensation appeared coordinated with metabolic alterations, as $\dot{M}_{O_2}$ was elevated when pHe compensation was occurring, but reduced when it was not. Whether these changes in $\dot{M}_{O_2}$ represent additional metabolic costs of pHe compensation through branchially-driven mechanisms, and thus imply energetic savings associated with preferential pHi regulation is still uncertain, but remains an intriguing possibility.

### 4.1. Hypercarbia and acid base physiology

Previous studies have demonstrated that white sturgeon exhibit no morbidity following rapid transfer to 45 mmHg $PCO_2$ within 48 h, and this was confirmed here. Additionally, 100% survival was observed following exposure to 60 and 90 mmHg $PCO_2$ at 24 and 6 h respectively despite a large increase in blood and tissue $PCO_2$ and a severe blood acidosis (up to 0.8 pH units; Figs. 1 and 5). Morbidity was observed in fish held at 60 and 90 mmHg for longer periods and at 90 mmHg PCO2 was associated with a decrease in HCT, [Hb], and

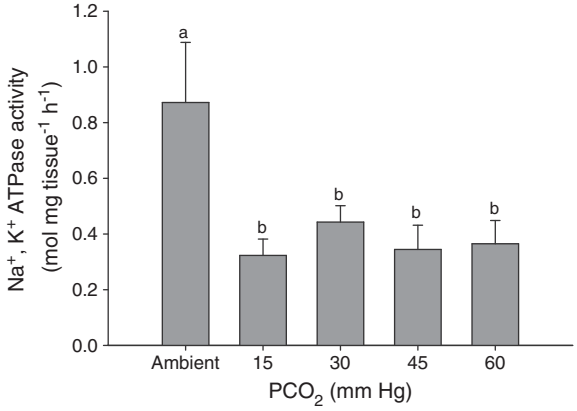


**Fig. 6.** The effect of 12 h of hypercarbia (normocarbia, 15, 30, 45, and 60 mmHg $PCO_2$) on maximal $Na^+$, $K^+$-ATPase activity of liver homogenates in white sturgeon. Values are means ± s.e.m. (n = 8 for each group, except 60 mmHg $PCO_2$ where n = 4). Dissimilar letters indicate a significant difference.

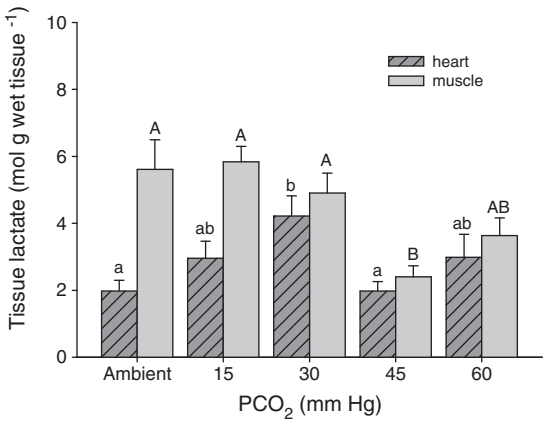


**Fig. 7.** The effect of 24 h of hypercarbia (normocarbia, 15, 30, 45, and 60 mmHg $PCO_2$) on lactate accumulation in heart (cross hatched bar) and white muscle (gray bars) in white sturgeon. Values are means ± s.e.m. (n = 8 for each group, except 60 mmHg $PCO_2$ where n = 4). Letters (lower case for heart, upper case for muscle) indicate significant differences between groups within a tissue type.

MCHC, and the decrease in $HCO_3^-$ (Table 1) implied that a loss of osmotic homeostasis may have preceded this. Long-term survival following direct transfer to a water $PCO_2$ of 45 mmHg has not been documented in any other exclusively water breathing fish (*i.e.*, non-air breathing), despite repeated observations in white sturgeon (Baker et al., 2009b). We have speculated that the exceptional $CO_2$ tolerance in white sturgeon, one of the greatest recorded to date, may be due to their great capacity for preferential pHi regulation during pHe depression (Brauner and Baker, 2009), which has now been observed *in vivo* (personal observations using magnetic resonance imaging, Baker et al., 2009a), *in situ* (Baker et al., 2011) and *in vitro* (Huynh et al., 2011).

In contrast, most vertebrates exhibit a qualitatively similar acidosis in the intra- and extracellular compartment during short term exposure to elevated $CO_2$ (*e.g.*, Rothe and Heisler, 1987; Wood et al., 1990; Wood and LeMoigne, 1991). Thus, survival in fishes during hypercarbia has traditionally been thought to rely at least partially on branchially-driven pHe compensation through net $HCO_3^-$ accumulation (Heisler, 1999; Brauner and Baker, 2009). Blood pH recovery, during more severe $CO_2$ challenges (>15 mmHg $PCO_2$ and ~0.4 pH units; Heisler, 1999) however remains incomplete and thus an intracellular acidosis persists. Despite their superior $CO_2$ tolerance, white sturgeon have similar pHe compensatory limits, and here exhibited full, partial or no pHe compensation as predicted at 15, 30 and ≥45 mmHg $PCO_2$ respectively within 48 h. The magnitude of blood pH recovery was closely associated with active net accumulation of plasma $HCO_3^-$ (in exchange for $Cl^-$; Fig. 3), suggesting compensatory mechanisms (*i.e.*, net $Cl^-/HCO_3^-$ exchange) similar to teleost fishes. White sturgeon liver and white muscle were not acidotic following 6 h of exposure to 45 and 90 mmHg $PCO_2$ (Fig. 1), instead exhibiting a slight increase in pHi. Previous studies have demonstrated that this regulation is not due to higher intrinsic buffering capacity of these tissues, but rather active pH regulatory response (Baker et al., 2009a). This unique combination of pHe compensation at low $CO_2$ levels but pHi protection independent of pHe compensation may allow further insight into the costs of acid–base regulation through examination of metabolic changes in response to different levels of hypercarbia.

### 4.2. $\dot{M}_{O_2}$ during hypercarbia

In white sturgeon, clearly metabolic demands changed according to the severity of aquatic hypercarbia to which these fish were exposed. A moderate increase in $CO_2$ (to 30 mmHg $PCO_2$) induced an overall increase in $\dot{M}_{O_2}$ (35%), which is similar to previous findings (increases of 30% during exposure to 20 mmHg $PCO_2$, (Crocker and Cech, 2002)). In response to a more severe hypercarbia (45 and 60 mmHg $PCO_2$), $\dot{M}_{O_2}$ decreased dramatically (~30 and 60%, respectively). Little comparative work exists for similar $CO_2$ tensions and exposure periods in other fish, but $CO_2$-sensitive species may increase $\dot{M}_{O_2}$ during small increases in environmental $PCO_2$ (*e.g.*, 5 mmHg $PCO_2$, *O. mykiss*, Thomas, 1983) which has not been observed in $CO_2$-tolerant species (~7 mmHg $PCO_2$, Deigweiher et al., 2008). In the $CO_2$-tolerant European eel, *Anguilla anguilla*, exposure to a stepwise increase in $CO_2$ induced a reduction in $\dot{M}_{O_2}$ at both 30 and 40 mmHg $PCO_2$ (Cruz-Neto and Steffensen, 1997). Thus, our findings suggest that the relationship between whole animal $\dot{M}_{O_2}$ and exposure to $CO_2$ may be different in sturgeon compared to other $CO_2$ tolerant fishes, but investigation into patterns associated with other fish species is needed.

### 4.3. What drives changes in metabolic rates during hypercarbia?

Metabolic rates can be altered as a result of changes in organismal, physiological and cellular processes, with some of these known to have large effects on aerobic or anaerobic respiration rates. However, overlaying changes in energetic costs or savings associated with methodological or hypercarbia-induced responses unrelated to acid–base physiology (*i.e.*, pH regulation) could significantly confound attempts to characterize metabolic changes associated with acid equivalent transport. In most fish, for example, a general stress response (*e.g.*, to handling) can induce elevations in metabolic rate. $\dot{M}_{O_2}$ in control fish in this study, was similar to that recorded in other studies (*e.g.*, Nonnotte et al., 1993), and did not change over the course of 48 h. Another possible confounding factor relates to the anesthetic effects of high $CO_2$, which are known to decrease whole animal $\dot{M}_{O_2}$. However, $CO_2$ levels used for anesthesia are typically much higher (135–760 mmHg $PCO_2$) than those used here, and survival tends to be low if exposure durations are greater than just a few minutes (Bernier and Randall, 1998). Consequently, both a general stress response and $CO_2$-induced anesthesia are not likely confounding sources of $\dot{M}_{O_2}$ adjustments observed in this study.

Changes in $\dot{M}_{O_2}$ observed during hypercarbia are not due to increased activity. Spontaneous activity decreased during exposure to hypercarbia, but overall was very low, and fish exhibited no sustained or burst swimming during $f_T$ assessment. Although tail beat frequency ($f_T$) more than doubled in control fish from 3 to 48 h, these higher rates were associated with no significant increase in $\dot{M}_{O_2}$ and swimming speeds lower than those of "resting" sturgeon in previous studies assessing factorial metabolic scope (Burggren, 1978; McKenzie et al., 2004; Geist et al., 2005). Therefore, we suggest that the reduction in $f_T$ at higher $CO_2$ levels is unlikely to account for much of the ~30 and ~60% reduction in $\dot{M}_{O_2}$ at 45 and 60 mmHg $PCO_2$ respectively.

Changes in $\dot{M}_{O_2}$ and $f_V$ were qualitatively matched, which is not surprising considering the link between ventilatory drive and $O_2$ demand. However, changes in ventilatory frequency observed here were modest compared to those seen in hypoxic or exercised sturgeons (McKenzie et al., 2004; Baker et al., 2005), and changes in ventilation amplitude were too small to be detected. Based on ventilatory costs of breathing in fish [hypothesized to be between 2 and 10% of routine metabolic rate at rest (Gilmour, 1998; Skovgaard and Wang, 2004), although likely low in white sturgeon (Burggren, 1978; Burggren and Randall, 1978)], we predict that the consequence of ventilatory changes observed here on $\dot{M}_{O_2}$ (Fig. 5) would be small, with the ~25% increase in $f_V$ at 30 mmHg $PCO_2$ and ~45% decrease in $f_V$ at 60 mmHg $PCO_2$ (Fig. 4.3B) resulting in no greater than a 5% increase or a 10% decrease in $\dot{M}_{O_2}$ respectively. Interestingly, other research (personal observation, Crocker et al., 2000; Baker et al., 2011) suggests that cardiovascular responses to severe hypercarbia in white sturgeon are also quite modest. Consequently, while we concede that ventilatory (but not behavioral) changes are likely contributing to the changes in $\dot{M}_{O_2}$ (60–80%) seen

here, we believe that they explain a small proportion relative to total changes.

The reductions in $\dot{M}_{O_2}$ during 45 and 60 mmHg $PCO_2$ could be linked to a decrease in cellular metabolism, or a switch to anaerobic processes for ATP production. White sturgeon in this study however, did not exhibit cellular responses supporting either of these possibilities. Specifically, maximal liver protein synthesis rates (suggested to account for 20–30% of total ATP-coupled $O_2$ demand) in white sturgeon liver homogenates were unaltered by hypercarbia, at least as assessed in the cell-free environment examined by this assay. Sturgeon may have a lower protein turnover rate based on a lower metabolic rate and reduce enzyme levels (although the relationship between protein turnover and metabolic rate in fish is still tenuous), and these findings are described in some detail elsewhere (Singer and Ballantyne, 2004). In other fishes investigated by a variety of methods including the one used in this study, protein turnover in liver can be decreased greatly (56–95%) within 12 h of anoxic or hypoxic challenge (Smith et al., 1996; Lewis et al., 2007; Jibb and Richards, 2008), and is associated with metabolic suppression and substantial metabolic savings (Bickler and Buck, 2007). *In vitro*, hepatocytes isolated from *Lepidonotothen kempi* only reduced protein synthesis rates in the presence of an acidosis, not elevated $CO_2$ (Langenbuch and Portner, 2003), but white sturgeon do not exhibit an acidosis in liver during hypercarbia (Fig. 1). On the other hand, a variety of regulatory mechanisms are potentially available to alter protein turnover rates, and remain to be investigated in sturgeons during hypercarbia. Changes in heart and white muscle lactate concentrations did not suggest any meaningful increase in anaerobic pathways (Fig. 7) after 24 h of hypercarbia.

Reducing cellular NKA activity represents another opportunity for metabolic saving as this transporter is estimated to account for as much as 25% of resting metabolic demand (Jackson, 2000), and is seen within 12 h of exposure to low oxygen in liver tissue of some hypoxia-tolerant vertebrates. In this study, maximal liver NKA activity was significantly reduced at all $CO_2$ tensions (Fig. 7). Thus, while the basis for this reduction remains to be explained, changes in potential NKA activity do not match the changes in $\dot{M}_{O_2}$ observed. Overall, cellular responses in white sturgeon did not indicate substantial metabolic depression or up-regulation of anaerobic respiration.

Changes in $\dot{M}_{O_2}$ during hypercarbia may reflect metabolic costs associated with acid–base regulation, such as the activation of branchial or cellular compensatory mechanisms. Current models of branchially-driven pH compensation during hypercarbia suggest roles for NHEs, V-ATPase, and NKA in driving acid excretion (Claiborne et al., 2002) which would be accompanied by an increase in ATP demand, and white sturgeon increase branchial expression of NKA during hypercarbia and have a relevant V-ATPase in the gills (Baker et al., 2009b). That the $\dot{M}_{O_2}$ increases during hypercarbia were concurrent with activation of mechanisms associated with net acid excretion (as assumed from pHe compensation in the blood) implies increased energetic expenditure. Factorial metabolic scope from $\dot{M}_{O_2}$ measured over 6 h periods was as high as 1.8 during hypercarbia, close to the 2-fold increases assessed in maximally exercised sturgeons (McKinley and Power, 1992; McKenzie et al., 2001; McKenzie et al., 2004; Geist et al., 2005; McKenzie et al., 2007). Thus, the increases in $\dot{M}_{O_2}$ induced by 15 and 30 mmHg $PCO_2$ are substantial for white sturgeon, although whether they reflect added costs associated with increased acid excretion through branchial mechanisms to drive pHe compensation remains to be determined.

Preferential pHi regulation has been hypothesized to represent a significant energetic savings, based simply on total base equivalents needing transport (Brauner et al., 2004; Brauner and Baker, 2009). During a hypercarbic challenge of 45 mmHg, for example, net extracellular $HCO_3^-$ accumulation corresponding with complete whole animal pH compensation (tissues and blood) would be approximately 130–150 mmol $L^{-1}$, although the tissues would require net $HCO_3^-$ accumulation of only 10–15 $mmol^{-1}$ for compensation. When adjusted to compensate for relative body fluid contribution (25% of total water being extracellular), we estimate that, in this example, greater than 7-fold more base equivalents would be required for whole animal compared to intracellular pH compensation. Even at low $CO_2$ tensions, preferential pHi regulation would require transport of considerably fewer base equivalents. Insight into the magnitude of these savings, while difficult to quantify, might be available from further study of the only two other fishes known to exhibit preferential pHi regulation [*i.e.*, *S. marmoratus* (Heisler, 1982) and *P. pardalis* (Brauner et al., 2004)], neither of which compensate for a blood acidosis during elevated $CO_2$.

## 5. Conclusions

The findings of this study provide empirical support for the hypothesis that preferential pHi regulation does not require substantial metabolic costs, and thus may represent an energetic savings over whole animal pH compensation. Further, we believe this work suggests that these metabolic savings could be significant enough to represent an advantage for which selection may have occurred in fishes exploiting environments prone to severe hypercarbia. While the mechanisms associated with intracellular pH protection in sturgeon remain to be elucidated, a shift to cellular acid extruding mechanisms that may be less energetically demanding has been observed in a few hardy fishes under some conditions (*e.g.*, Krumschnabel et al., 2001). We hope our observation that preferential pHi regulation can occur during substantial decreases in metabolic rate may provide guidance for studies focusing on elucidating cellular mechanisms responsible for preferential pHi regulation.

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