PHYSIOLOGICAL RESPONSES ASSOCIATED WITH AQUATIC HYPERCARBIA IN THE CO_2-TOLERANT WHITE STURGEON, ACIPENSER TRANSMONTANUS

by

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ABSTRACT

Through investigations conducted at the organismal, tissue and cellular levels, this thesis provides clear evidence that the white sturgeon, *Acipenser transmontanus*, is among the most CO_2 tolerant of all fishes investigated to date. During moderate increases in water CO_2 tension (PCO_2) (≤ 15 mm Hg PCO_2, hypercarbia), white sturgeon exhibited changes in gill morphology and restored blood pH (pHe) through net HCO_3^-/Cl^-, a process observed in most fishes (Chapter 3). At CO_2 tensions lethal to other fishes (≥ 22.5 mm Hg PCO_2), white sturgeon completely protected intracellular pH (pHi) of the heart, liver, brain and white muscle (termed preferential pHi regulation), despite a large reduction in pHe (up to 1 pH unit) (Chapter 3, 4). Tissue pHi regulation was activated in heart within minutes of the onset of hypercarbia (measured *via* NMR, Chapter 5), and completely protected pHi in this tissue even during exposure to potentially lethal CO_2 levels (i.e., 90 mm Hg PCO_2). In hearts examined *in situ*, maximum cardiac performance was well defended and associated with partial pHi compensation in ventricles (which exhibited only ~40% of predicted acidosis). Preferential pHi regulation was not associated with large increases in metabolic costs, as during exposure to severe hypercarbia (~45 mm Hg PCO_2), heart [ATP] and [CrP] had recovered to pre-exposure levels within 90 min, and whole animal $\dot{M}_{O_2}$ was decreased (30%) when pHi was completely protected. Preferential pHi regulation of this magnitude and rapidity has not been documented before in any vertebrate in response to hypercarbia and represents a novel pattern of acid-base regulation among fishes.

White sturgeon represent the first exclusively water-breathing fish to exhibit preferential pHi regulation during hypercarbia. Furthermore, white sturgeon are the most basal vertebrate to demonstrate complete pHi protection during severe pHe depression. As sturgeon may retain ancestral characteristics, I propose that preferential pHi regulation is the basis for enhanced CO_2 tolerance in other tolerant Osteichthyan fishes, and first arose in association with ionoregulatory and respiratory challenges experienced during freshwater invasion in the vertebrate lineage.

PREFACE

Chapter One: General Introduction

Comments: This chapter was written by Daniel W. Baker under the supervision of Dr. Colin J. Brauner who also supplied editorial advice. This chapter draws on the book chapter co-authored by C. J. Brauner and Daniel W. Baker entitled "Patterns of acid-base regulation in fish" in *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects* (edited by M. L. Glass, and S. C. Wood. Berlin, Germany: Springer-Verlag, 2009).

Chapter Two: A validation of intracellular pH measurements in fish exposed to hypercarbia: The effect of duration of tissue storage and efficacy of the metabolic inhibitor homogenate method

Comments: All aspects of this study were designed, conducted and written by Daniel W. Baker, and under the supervision of Dr. Colin J. Brauner who also supplied editorial comments on the chapter. Intracellular pH measurements were made with the aid of a directed studies student, T. May. A version of this chapter has been published as a short communication by Daniel W. Baker as first author and T. May and C. J. Brauner as co-authors entitled "A validation of intracellular pH measurements in fish exposed to hypercarbia: The effect of duration of tissue storage and efficacy of the metabolic inhibitor tissue homogenate method" in the Journal of Fish Biology, 75(1): 268-275, 2009. T. May performed a portion of the experimental work under D. Baker's supervision

Chapter Three: Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon

Comments: This chapter was written by Daniel W. Baker under the supervision of Dr. Colin J. Brauner. All experiments were designed and carried out or directly supervised by Daniel W. Baker. C. J. Brauner provided valuable supervision and provided useful comments on the completed chapter. A version of this chapter has been published by Daniel W. Baker as first author and co-authors V. Matey, K. T. Huynh, J. M. Wilson, J. D. Morgan and C. J. Brauner entitled "Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon." American Journal of Physiology Regulatory Integrative and Comparative Physiology 296:1868-1880, 2009. All authors provided editorial contributions. V. Matey supplied electron microscopic images and measurements. K.T. Huynh performed a portion of the experimental work. J. M. Wilson was responsible for immunohistology and enzyme assays. J. D. Morgan provided experimental and editorial advice, and access to fish.

Chapter Four: Metabolic effects of aquatic hypercarbia in the CO_2-tolerant chondrostean, white sturgeon.

Comments: All aspects of this study were designed, conducted and written by Daniel W. Baker., and under the supervision of Dr. Colin J. Brauner who also supplied editorial comments on the chapter.

Chapter Five: The exceptional intracellular pH regulatory response of white sturgeon, *Acipenser transmontanus*, during hypercarbia is activated rapidly *in vivo*: A ^{31}P-NMR study.

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Chapter Six: Exceptional protection of maximum cardiac performance during hypercapnia is further enhanced by adrenergic stimulation in perfused hearts of the CO_2-tolerant white sturgeon.

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Chapter Seven: General Discussion

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TABLE OF CONTENTS

ABSTRACT ..... **ii**

PREFACE ..... **iii**

TABLE OF CONTENTS ..... **v**

LIST OF TABLES ..... **x**

LIST OF FIGURES ..... **xi**

LIST OF SYMBOLS AND ABBREVIATIONS ..... **xviii**

ACKNOWLEDGEMENTS ..... **xxi**

DEDICATION ..... **xxiii**

1: GENERAL INTRODUCTION **1**

1.1 OVERVIEW 1

1.2 ACID-BASE REGULATION IN FISHES 2

1.3 THE IMPORTANCE OF INVESTIGATING AQUATIC HYPERCARBIA 4

1.4 PHYSIOLOGICAL RESPONSES OF FISHES TO AQUATIC HYPERCARBIA 5

1.5 LIMITATIONS TO PHE COMPENSATION DURING AQUATIC HYPERCARBIA 9

1.6 CO_2-TOLERANT FISHES AND THE CHALLENGE OF SEVERE HYPERCARBIA 11

1.7 WHITE STURGEON AS A REPRESENTATIVE SPECIES FOR CO_2-TOLERANT FISHES 12

1.8 THESIS OBJECTIVES AND OUTLINE 14

2: A VALIDATION OF INTRACELLULAR PH MEASUREMENTS IN FISH EXPOSED TO HYPERCARBIA: THE EFFECT OF TISSUE STORAGE DURATION AND EFFICACY OF THE METABOLIC INHIBITOR TISSUE HOMOGENATE METHOD **21**

2.1 SYNOPSIS 21

2.2 INTRODUCTION 21

2.3 MATERIALS AND METHODS 25

2.3.1 Animals and rearing conditions 25

2.3.2 Series 1: The effect of storage duration and method on pHi 25

2.3.3 Series 2: The effect of CO_2 tension on pHi measurements 26

2.3.4 Intracellular pH measurements 27

2.3.5 Statistical analyses 27

2.4 RESULTS 28

2.4.1 Series 1: The effect of storage duration and method on pHi 28

2.4.2 Series 2: The effect of CO_2 tension on pHi measurements 28

2.5 DISCUSSION 29
3: COMPLETE INTRACELLULAR pH PROTECTION DURING EXTRACELLULAR pH DEPRESSION IS ASSOCIATED WITH HYPERCARBIA TOLERANCE 34
3.1 SYNOPSIS 34
3.2 INTRODUCTION 35
3.3 MATERIALS AND METHODS 37
3.3.1 Animals and rearing conditions 37
3.3.2 Series 1: The effect of hypercarbia (11.5 mm Hg PCO_2) on blood and tissues 38
3.3.3 Series 2: The effect of hypercarbia (22.5 and 45 mm Hg PCO_2) on blood and tissues 39
3.3.4 Analytical techniques 40
3.3.5 Scanning electron microscopy 42
3.3.6 ATPase assay 42
3.3.7 Immunoblotting 43
3.3.8 Immunofluorescence microscopy 43
3.3.8 Statistical analyses 44
3.4 RESULTS 45
3.4.1 Series 1: The effect of hypercarbia (11.5 mm Hg PCO_2) on blood and tissues 45
3.4.2 Series 2: The effect of hypercarbia (22.5 and 45 mm Hg PCO_2) on blood and tissues 47
3.5 DISCUSSION 49
3.5.1 White sturgeon during normocarbia 49
3.5.2 pHe recovery during moderate hypercarbia 50
3.5.3 pHi during hypercarbia exposure 53
3.5.4 Conclusions 56
4: METABOLIC EFFECTS OF AQUATIC HYPERCARBIA 71
4.1 SYNOPSIS 71
4.2 INTRODUCTION 72
4.3 MATERIALS AND METHODS 74
4.3.1 Animals and rearing conditions 74
4.3.2 Experimental protocols 75
4.3.2.1 Series 1: The effect of hypercarbia on survival, haematology and acid-base physiology 75
4.3.2.2 Series 2: The effect of hypercarbia on oxygen consumption rate ($\dot{M}_{O_2}$) 77

4.3.2.3 Series 3: The effect of hypercarbia on energetically-relevant parameters; tail beat and ventilation frequency, cell-free protein synthesis rate and tissue lactate levels. 78
4.3.3 Statistical analyses 80
4.4 RESULTS 81
4.4.1 Series 1: The effect of hypercarbia on survival, haematology and acid-base physiology 81
4.4.2 Series 2: The effect of hypercarbia on oxygen consumption rate ($\dot{M}_{O_2}$) 81
4.4.3 Series 3: The effect of hypercarbia on energetically-relevant parameters; tail beat and ventilation frequency, cell-free protein synthesis rate and tissue lactate levels 82
4.5 DISCUSSION 83
4.5.1 Hypercarbia survival and acid base regulation 84
4.5.2 Oxygen consumption rate during moderate and severe hypercarbia 86
4.5.3 Changes in metabolic demands during severe hypercarbia 89
4.5.4 Conclusions 91
5. *IN VIVO* INTRACELLULAR pH AND METABOLIC RESPONSES DURING SEVERE SHORT TERM AQUATIC HYPERCARBIA: A ^{31}P NMR STUDY 101
5.1 SYNOPSIS 101
5.2 INTRODUCTION 102
5.3 METHODS AND MATERIALS 104
5.3.1 Animals and rearing conditions 104
5.3.2 Protocol for hypercarbia exposure 105
5.3.2.1 Series 1: The effect of hypercarbia on the heart *in vivo* using ^{31}P NMR spectra acquisition 105
5.3.2.2 Series 2: The effect of hypercarbia on the heart, white muscle and RBC 105
5.3.3 Analytical techniques 106
5.3.3.1 NMR imaging and spectroscopy 106
5.3.3.2 Analytical techniques on excised tissues 108
5.3.4 Calculations 108
5.3.5 Statistical analyses 110
5.4 RESULTS 111
5.4.1 The effect of hypercarbia on tissue pHi 111
5.4.2 The effect of hypercarbia on tissue metabolites 112

5.5 DISCUSSION 112
5.5.1 Interpretation of ^{31}P NMR spectra 113
5.5.2 The effect of short-term hypercarbia on heart pHi 114
5.5.3 The effect of short-term aquatic hypercarbia on metabolites in the heart 116
5.5.4 The pHi regulatory response of white sturgeon hearts 117
5.5.5 Conclusions 119
6: EXCEPTIONAL PROTECTION OF MAXIMUM CARDIAC PERFORMANCE DURING HYPERCAPNIA IS FURTHER ENHANCED BY ADRENERGIC STIMULATION IN PERFUSED HEARTS 127
6.1 SYNOPSIS 127
6.2 INTRODUCTION 128
6.3 METHODS AND MATERIALS 130
6.3.1 Animals and rearing conditions 130
6.3.2 Surgical procedures 130
6.3.3 Perfusate composition 132
6.3.4 Experimental protocols 133
6.3.4.1 Series 1: The effect of hypercapnia (22.5, 45, and 60 mm Hg PCO_2) on maximum cardiac performance 134
6.3.4.2 Series 2: The effect of hypercapnia (45 mm Hg PCO_2) on subsequent recovery of maximum cardiac performance 134
6.3.4.3 Series 3: The effect of hypercapnia (45 mm Hg PCO_2) on maximum cardiac performance with maximal exogenous stimulation by adrenaline (500 nmol l^{-1} [AD]) 135
6.3.5 Tissue pHi determination 135
6.3.6 Calculations and statistical analyses 136
6.4 RESULTS 137
6.4.1 Series 1: The effect of hypercapnia (22.5, 45, and 60 mm Hg PCO_2) on maximum cardiac performance 137
6.4.2 Series 2: The effect of hypercapnia (45 mm Hg PCO_2) on subsequent recovery of maximum cardiac performance 137
6.4.3 Series 3: The effect of hypercapnia (45 mm Hg PCO_2) on maximum cardiac performance with maximal exogenous stimulation by adrenaline (500 nmol l^{-1} [AD]) 138
6.4.4 Tissue pHi determination 138

6.5 DISCUSSION 139
6.5.1 Maximum cardiac performance during hypercapnia 139
6.5.2 Protective effects of adrenergic stimulation on cardiac performance during hypercapnia 141
6.5.3 Conclusions 143
7: GENERAL DISCUSSION 151
7.1 A VALIDATION OF THE TISSUE HOMOGENATE METHOD OF PHI ASSESSMENT FROM TISSUES EXPOSED TO HYPERCARBIA 151
7.2 PREFERENTIAL PHI REGULATION IS ASSOCIATED WITH CO_2 TOLERANCE 152
7.3 PREFERENTIAL PHI REGULATION IS NOT ASSOCIATED WITH INCREASES IN WHOLE ANIMAL METABOLIC RATE 153
7.4 PREFERENTIAL PHI REGULATION IS RAPIDLY ACTIVATED DURING HYPERCARBIA 154
7.5 CARDIAC PERFORMANCE IS EXCEPTIONALLY TOLERANT OF HYPERCARBIA AND ACIDOSIS 155
7.6 PREFERENTIAL PHI REGULATION AS A STRATEGY FOR CO_2 TOLERANCE 156
7.7 EVOLUTIONARY SIGNIFICANCE 159
7.7.1 Survey of extant primitive fishes 160
7.7.1.1 Agnathans (hagfishes and lampreys) 161
7.7.1.2 Chondrichthyans (sharks, batamorphs and chimaeriformes) 162
7.7.1.3 Sarcopterygiians (lungfishes, coelacanth and tetrapods) 163
7.7.1.4 Basal actinopterygiians 163
7.7.1.5 Teleosts 165
7.7.2 The origin of preferential pHi regulation 166
7.7 FINAL THOUGHTS 170
BIBLIOGRAPHY 177

LIST OF TABLES

Table 3.1 The effect of short-term (6, 24, and 48 h) hypercarbia (11.5 mm Hg PCO_2) on plasma ion status and blood glucose in white sturgeon. Values are means ± s.e.m. Dissimilar letters signify discrete subsets, and thus letters indicate significant difference among treatments. 58

Table 3.2 The effect of short-term (48 h) hypercarbia (Series 1, 11.5 mm Hg PCO_2; Series 2, 22.5 and 45 mm Hg PCO_2) on tissue intracellular [HCO_3^-] in white sturgeon. Values are means ± s.e.m. An asterisk indicates significant difference from respective control treatment. 59

Table 4.1 The effect of short-term (24 h) hypercarbia (45 and 90 mm Hg PCO_2) on haematocrit (HCT, %), haemoglobin concentration (Hb; mmol l^{-1}), mean cell haemoglobin concentration (MCHC), plasma bicarbonate concentration (mmol l^{-1}), and plasma chloride concentration (mmol l^{-1}) in white sturgeon. Values are mean ± s.e.m. An asterisk indicates a significant difference from the control treatment. 92

Table 5.1 The effect of short term (90 min) of hypercarbia (45 mm Hg PCO_2) on intracellular pH (pHi), ATP, creatine phosphate (CrP) and free creatine (Cr) in red blood cells (RBC), heart and white muscle of white sturgeon as measured on excised tissues. Values are means ± s.e.m. (N = 5-7 for each group). An asterisk indicates a significant difference from control treatment. 120

Table 6.1 The effect of hypercapnia (22.5, 45 or 60 mm Hg PCO_2) and maximal adrenergic stimulation (45 mm Hg PCO_2 and 500 nmol l^{-1} [AD]) on rate of ventricular force generation (F_V) in perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. An asterisk indicates a statistically significant difference from normocapnia expose hearts within a given PCO_2 treatment..... 145

Table 6.2 The effect of hypercapnia (45 mm Hg PCO_2) and maximal adrenergic stimulation (500 nmol g^{-1} [AD]) on white sturgeon ventricular intracellular pH (pHi). *In vivo* values were obtained from ventricles excised from white sturgeons under resting conditions. Control group represents ventricles sampled during 3.75 mm Hg PCO_2. Values are mean ± s.e.m. Letters indicate significant differences among treatments. 146

LIST OF FIGURES

Figure 1.1 The effect of short-term (24 h) of low hypercarbia (7 mm Hg PCO_2) on blood pH and plasma HCO_3^- in rainbow trout as represented on a pH/HCO_3^-/CO_2 plot. Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point represent exposure time; arrows indicate temporal pattern of change in blood pH and plasma HCO_3^-. The dotted line indicates the intrinsic (i.e., non-bicarbonate) buffer value of whole blood as reported by Wood and LeMoigne (1991) (see text for further details). Data from Larsen and Jensen (1997). 18

Figure 1.2 The relationship between the pH of blood (pHe) and intracellular pH (pHi) of red blood cells (open circles), brain (filled circles), and white muscle (inverted triangle) during exposure to short term low hypercarbia in (A) little skate, *R. ocellata*, and (B) hyperoxia-induced hypercarbia in rainbow trout, *O. mykiss*. Time course (h) is indicated by numbers located directly above vertically oriented groupings. Note reversal of early time points (0.5 and 2 h) in panel A. While pHi can recover more rapidly than pHe in tissues such as the brain and heart of skate, in all tissues presented here, pHi remains depressed if pHe does not recover. Mean values and s.e.m. bars approximated from Wood et al. (1990) for skate and Wood and LeMoigne (1991) for trout. 19

Figure 1.3 A theoretical representation of the "typical" temporal response to short-term (less than 96 h) hypercarbia in fish. Upon transfer from normocarbia to hypercarbia, blood pH rapidly falls along the non-bicarbonate buffer line of the blood as indicated by black open arrowhead, and pH recovers along a given PCO_2 isopleth through a net increase in HCO_3^- in exchange for Cl^- as indicated by black filled arrowheads. Black filled circles represent final pHe values that would be achieved based upon apparent limits to net HCO_3^- accumulation within 24-96 h of exposure to hypercarbia (see text for further details). Shaded bar indicates maximal pH compensation limited by the "bicarbonate concentration threshold", as most fish do not increase plasma HCO_3^- beyond 25-35 mmol l^{-1} (modified from Heisler 1986, 1999). Thus, compensation for a respiratory acidosis (within 48-96h) during exposure to hypercarbia is incomplete above a PCO_2 of 10-15 mm Hg in most water-breathing fishes. Note that CO_2 isopleths are dependent on both temperature and osmolarity, and that the isopleths represented here are plotted for clarity purposes only. 20

Figure 2.1 The effect of duration (days) of storage in liquid nitrogen (LN_2) on red blood cell (RBC) pHi in white sturgeon exposed to 30 mm Hg PCO_2 for 48 h. The group furthest to the right represents the mean pHi value of RBC frozen in LN_2 and transferred immediately to an ultracold (-80°C) freezer for 90 days. Values are means ± s.e.m. An asterisk indicates a significant difference from groups measured prior to storage. RBC pHi was measured *via* the freeze-thaw method (FAT) of Zeidler and Kim (1977). 31

Figure 2.2 The relationship between pHe and pHi in response to different levels of CO_2 as described by either the freeze-thaw method (FAT) (circles) or the metabolic inhibitor tissue homogenate (MITH) method (triangles). Raw data are shown. The slopes and the elevations of the regression lines for FAT (dashed line; $m = 0.66$, $r^2 = 0.946$) and MITH (solid line; $m = 0.58$, $r^2 = 0.972$) are not significantly different from each other. 32

Figure 2.3 The correlation between pHi measured *via* the freeze-thaw method (FAT) or the metabolic inhibitor tissue homogenate (MITH) method. The slope of the line for this correlation is not significantly different from 1 ($m = 0.84$, $r^2 = 0.945$, $p < 0.001$). 33

Figure 3.1 The effect of short-term (6, 24, and 48 h) hypercarbia (11.5 mm Hg PCO_2) on A) arterial pH and plasma [HCO_3^-] (mmol l^{-1}) presented as a pH/HCO_3^-/CO_2 diagram, B) red blood cell (RBC) pHi, and C) brain (circle), liver (triangle), and heart (square) pHi in white sturgeon, *A. transmontanus*. Values (n=6-7) are presented as means ± s.e.m. In A), time (h) is indicated next to each point, the dotted line represents the blood non-bicarbonate buffer line, a dagger indicates a significant change in pH, and an asterisk indicates significant change in plasma [HCO_3^-] from normocarbia (control). In B) and C), an asterisk indicates a significant difference from normocarbia (control). 60

Figure 3.2 Microstructure of A) the epithelium covering the trailing edge (TE) of gill filaments (scale bar: 100 μm), B) pavement cells (PVC), mucous cells (MC), and mitochondrial-rich cells with large apical surface area (MRCLA, white arrows) and smaller surface area (MRCSA, blackhead arrows) (scale bar: 10 μm), C) long and thin microvilli representative of MRCLA (scale bar: 2 μm) and D) short and thick microvilli representative of MRCSA (scale bar: 2 μm) on the surface of gill filament epithelium in white sturgeon exposed to normocarbia. 61

Figure 3.3 Ultrastructure of filament epithelium in gills of white sturgeon following exposure to A) normocarbia for 48 h, or moderate hypercarbia (11.5 mm Hg PCO_2) for B) 6 h, C) 24 h, and D) 48 h. MRCLA are indicated with whitehead arrows (note absence in B-D), MRCSA with blackhead arrows (scale bars: 5 μm). Apical ultrastructure of MRCSA during exposure to E) normocarbia for 48 h, and hypercarbia (11.5 mm Hg PCO_2) for F) 24 h and G) 48 h under greater magnification (scale bars: 1μm). 62

Figure 3.4 The effect of short-term (6, 24, and 48 h) moderate hypercarbia (11.5 mm Hg PCO_2) on A) pavement cell microridge density (intercepts $grid^{-1}$), B) mitochondrial-rich cell (MRC) density (number mm^{-2}), C) MRC surface area (μm^2), and D) MRC fractional area (FAMRC; % epithelium $unit^{-1}$) on the filament epithelium in the white sturgeon. Values are presented as means ± s.e.m. (n = 6-7). Letters indicate significant differences between groups. 63

Figure 3.5 The effect of short-term (6, 24, and 48 h) moderate hypercarbia (11.5 mm Hg PCO_2) on activity of either A) branchial Na^+,K^+-ATPase (NKA) activity (μmol ADP mg^{-1} $protein^{-1}$), B) V-ATPases activity (μmol ADP mg^{-1} $protein^{-1}$), or expression of C) α subunit of NKA, D) B subunit of V-ATPase, or E) NHE3 in representative western blots in white sturgeon. In A and B, values are presented as means ± s.e.m. (n=6-7). Letters indicate significant differences between groups. 64

Figure 3.6 Indirect immunofluorescent localization of Na^+,K^+-ATPase α subunit (A, D) with either (B) NHE3 or V-ATPase B subunit (E) in normocarbic sturgeon gill sections (scale bar: 20 μm). Merged images of counter stained (DAPI, blue) sections were overlaid for tissue orientation (C, F). Arrowheads (A-C) indicate NHE3 immunoreactive (IR) cells, arrows (D-F) indicate V-ATPase IR cells, crossed arrows (D-F) indicate cells that double label with V-ATPase and Na^+,K^+-ATPase, and asterisks indicate erythrocytes. Moderate hypercarbia (11.5 mm Hg PCO_2 for 48 h) did not qualitatively alter the staining patterns of either NHE3 or V-ATPase (data not shown). 65

Figure 3.7 The effect of short-term (48 h) severe hypercarbia (normocarbia, circles; 22.5 mm Hg PCO_2 squares; or 45 mm Hg PCO_2 , inverted triangles) on blood pH and plasma [HCO_3^-] in cannulated white sturgeon. Blood pH is plotted as A) a function of time, sampled at 15 and 30 minutes, and 1, 3, 6, 12, 24 and 48 h, and B) against plasma [HCO_3^-], represented on a pH/HCO_3^-/CO_2 plot. Values are presented as means ± s.e.m. (n=4-6). In A), letters indicate differences between groups. In B), numbers on figure indicate time in hours, and dotted line indicates intrinsic buffer line for blood oriented through normocarbic data (normocarbic data presented in A, not shown in B for clarity). 66

Figure 3.8 The effect of short-term (48 h) severe hypercarbia (normocarbia, circles; 22.5 mm Hg PCO_2 squares; or 45 mm Hg PCO_2 , inverted triangles) on red blood cell (RBC) intracellular pH (pHi) as a function of A) time or B) blood pH in cannulated white sturgeon. In A), values are presented as means ± s.e.m. (only groups where n > 3 are presented), and different letters indicate time points within a treatment that are significantly different. In B), the correlation between blood pH (pHe) and RBC pHi was significant (slope = 0.52, r^2 = 0.90, $p < 0.05$). 67

Figure 3.9 Relationship between blood extracellular pH (pHe) and intracellular pH (pHi) of RBC (circles), white muscle (squares) and liver (inverted triangle) (A) and heart (circles) and brain (inverted triangles) (B) of white sturgeon following 48 h of exposure to either normocarbia (air-equilibrated water) or severe hypercarbia (22.5 and 45 mm Hg PCO_2). Tissues are presented in separate panels for clarity. Values are presented as means ± s.e.m. (n = 4-6). Correlations between raw data for pHe and tissue pHi are described by the following lines: RBC: m = 0.48, $r^2 = 0.96$, $P < 0.05$; heart: slope 0.14, $r^2 = 0.67$, $P < 0.05$; brain: slope = 0.24, $r^2 = 0.72$, $P < 0.05$; 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). 68

Figure 3.10 Relationship between blood pH (pHe) and plasma [HCO_3^-] in blood equilibrated *in vitro* at 3.75, 7.5, 15, 30, 45, and 75 mm Hg PCO_2. Values are means ± s.e.m. (n = 4). Intrinsic buffer capacity of blood (β_{NB} = -11.9 mmol HCO_3^- mmol l^{-1} pH $unit^{-1}$, $r^2 = 0.878$) was calculated from the slope of the best-fit linear regression over *in vivo* pHi values..... 69

Figure 3.11 Relationship between pH and [HCO_3^-] in tissue homogenates prepared from white muscle (A), heart (B), and liver (C), equilibrated at 3.75, 7.5, 15, and 30 mm Hg PCO_2. Values are means ± s.e.m. (n = 6-8). Intrinsic buffering of these homogenates (β_{NB}) were calculated from the slope of the best-fit regression (white muscle, $r^2 = 0.85$; heart, $r^2 = 0.78$; liver, $r^2 = 0.13$) over *in vivo* relevant pHi values, and tissue buffer capacity was calculated from these values (see text for details). 70

Figure 4.1 The effect of short-term (96 h) hypercarbia (45 mm Hg PCO_2, filled circles; 60 mm Hg PCO_2, open circles; 75 mm Hg PCO_2, inverted filled triangles; and 90 mm Hg PCO_2, open triangles) on white sturgeon survival (%). Values represent means ± s.e.m. (n = 4-8, with 10 fish per tank). An asterisk indicates a difference between the associated treatment and ambient PCO_2 treatment at a given sampling time. Mean survival of fish exposed to normocarbia, 15, and 30 mm Hg PCO_2 was 100% at all time points and data were removed for clarity. 93

Figure 4.2 The effect of short term (6 h) hypercarbia (normocarbia, white bars; 45 mm Hg PCO_2, light bars; 90 mm Hg 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 = 9). An asterisk indicates a statistically significant difference from normocarbia exposed group. 94

Figure 4.3 The effect of short-term (48 h) hypercarbia on A) overall mean oxygen consumption rate ($\dot{M}_{O_2}$) and B) oxygen consumption rate (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) over time (pooled in 6 h periods) of white sturgeon. Panel C shows a representative trace of a fish during normocarbia. Values are means ± s.e.m. (n = 8 for each treatment). An asterisks indicates a significant difference from normocarbic treatment at a given sampling time. Letters indicate significant differences within a treatment. At 60 mm Hg, only three fish survived 36 h, and none survived past 45 h. 95

Figure 4.4 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on tail beat frequency (f_T) of white sturgeon. Values are means ± s.e.m. (n = 6-10 for each treatment). There is an overall effect of CO_2 treatment and time, but no significant interaction. Letters indicate differences between main treatment effects of CO_2. At 60 mm Hg, no fish survived 48 h. 96

Figure 4.5 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on ventilation frequency (f_V) of white sturgeon. Values are means ± s.e.m. (n = 4-8 for each value). There is an overall effect of CO_2 treatment and time, but no significant interaction. Letters indicate differences between main treatment effects of CO_2. At 60 mm Hg, no fish survived 48 h..... 97

Figure 4.6 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on A) whole blood pH and B) plasma [HCO_3^-] of white sturgeon. Values represent mean ± s.e.m. (n=4-7 per group). Letters 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 mm Hg, no fish survived 48 h. 98

Figure 4.7 The effect of short-term (12 h) hypercarbia (normocarbia, 15, 30, 45, and 60 mm Hg PCO_2) on A) maximal protein synthesis rate and B) maximal Na^+, K^+-ATPase activity of liver homogenates in white sturgeon. Values are means ± s.e.m. (n = 4-8 for each group). Dissimilar letters indicate a significant difference. No statistically significant differences were observed in protein synthesis rates. 99

Figure 4.8 The effect of short-term (24 h) hypercarbia (normocarbia, 15, 30, 45, and 60 mm Hg PCO_2) on lactate accumulation in heart (cross hatched bar) and white muscle (grey bars) in white sturgeon. Values are means ± s.e.m. (n = 5-8 for each group). Letters (lower case for heart, upper case for muscle) indicate significant differences between groups within a tissue type. 100

Figure 5.1 Representative 2 dimensional ^1H NMR images collected from white sturgeon. Panel A shows a longitudinal section and illustrates the location of the heart within the fish for precise positioning of the proton and phosphate NMR coils. Panel B is an axial section

(vertical slice) centered on the heart of the white sturgeon, and images such as these were used to prescribe the saturation slice and eliminate signal from this area. Panel C shows an estimation of the signal area (the semi-circle outlined with a white line) and the saturation slice (area of removed signal, darkened area within the semi-circle), clearly demonstrating the increase in the proportion of signal coming from the heart (see text for further details). 121

Figure 5.2 Representative ^{31}P-NMR spectra obtained from the signal matched to the ^{1}H NMR image centered on the heart of white sturgeon *in vivo*. ATPα, adenosine triphosphate (as implied by *alpha* phosphate group); CrP, creatine phosphate; Pi, intracellular phosphate. Only peaks quantified for use within the present study are identified, for clarity purposes. 123

Figure 5.3. Water PCO_2 following initiation of hypercarbia within the chamber used to hold white sturgeon during ^{31}P-NMR spectra acquisition. Data is presented as mean ± s.e.m. (n = 3 for each data point). Dotted line represents PCO_2 of gas mixture used for aeration during aquatic hypercarbia. 124

Figure 5.4 The effect of 90 min exposure to severe hypercarbia on intracellular pH of the heart in white sturgeon *in vivo* calculated from ^{31}P NMR. Values are expressed as the absolute change in pHi (ΔpHi) relative to normocarbic controls (time 0 values), and are calculated from pooled values of all 2.5 min scans collected over 15 min. Time zero represents the point at which aeration with pre-mixed gas began. Values are mean ± s.e.m. (n = 4 for each data point). An asterisk indicates significant differences from pre-exposure group (one-way RM ANOVA, p = 0.025). 125

Figure 5.5 The effect of short-term (90 min) hypercarbia (45 mm Hg PCO_2) on relative levels of A) ATP, B) CrP and C) ADP_{free} in white sturgeon hearts *in vivo* calculated from ^{31}P NMR. CrP levels are normalized to the sum of CrP and Pi and ATP, CrP and ADP_{free} are expressed relative to pre-exposure (i.e., time 0) values, the point at which aeration was switched to 45 mm Hg PCO_2 gas. Values are mean ± s.e.m. (n = 4 for each data point). An asterisk indicates a significant difference from pre-exposure values. 126

Figure 6.1 The effect of hypercapnia (22.5, 45 and 60 mm Hg PCO_2) on A) heart rate (f_H), B) stroke volume (V_S), C) maximum cardiac output (Q_{max}), and D) maximum cardiac power output, (PO_{max}), expressed as a percentage of control values assessed on perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. An asterisk indicates a statistically significant change from control values within that CO_2 tension. Dotted line represents control values (i.e., 100%) for comparative purposes. 147

Figure 6.2 A diagram representing the effect of hypercapnia (45 and 60 mm Hg PCO_2) on heart beat interval (time in seconds between beats) during cardiac performance testing. The top two panels (A and B) are data from an *in situ* perfused heart sequentially exposed to A) 3.75 mm Hg PCO_2 and then B) 45 mm Hg PCO_2. The bottom two panels (C and D) are data from an *in situ* perfused heart sequentially exposed to C) 3.75 mm Hg PCO_2 and then D) 60 mm Hg PCO_2. Note the bimodal distribution of long and short heat beat intervals in the heart preparation exposed to 60 mm Hg PCO_2 (Panel D). 148

Figure 6.3 The effect of hypercapnia (45 mm Hg PCO_2) and return to control CO_2 tension ("rec"; 3.75 mm Hg PCO_2) on A) heart rate(f_H), B) stroke volume (V_S), C) maximum cardiac output (Q_{max}) and D) maximum cardiac power output (PO_{max}) assessed on perfused

white sturgeon hearts *in situ*. Values are means ± s.e.m.(n = 6). Letters indicate statistically significant differences between treatment groups. 149

Figure 6.4 The effect of hypercapnia (45 mm Hg PCO_2) in the absence and presence of adrenaline (6 w/AD; 500 nmol l^{-1} [AD]) on A) heart rate, f_H, B) stroke volume, V_S, C) maximum cardiac output, Q_{max}, and D) maximum cardiac power output, PO_{max}, assessed on perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. (n = 8). Letters indicate statistically significant differences between treatment groups. 150

Figure 7.1 Net acid equivalent removal required to recover normocarbic pH in the whole body (WB) (i.e., extra- and intracellular compartments), extracellular (EC), or intracellular (IC) compartments ofa 1 kg fish at 12°C during exposure to A) 7.5 mm Hg PCO_2 and B) 45 mm Hg PCO_2. In B, whole body total chloride ion content (WB Cl^-) is also indicated to illustrate counter ion exchange limitations. CO_2 solubility and equilibrium constants are calculated from previously determined equations (Boutilier et al., 1984). Note differing scales on y-axis between A and B. 171

Figure 7.2 A summary of acid-base relevant physiological and behavioural characteristics overlaid on a phylogenetic representation of the interrelatedness within the craniate lineage, using the topology that is most widely accepted by morphologists and palaeontologists. Within each taxon, "CO_2 tolerance" refers to whether there are examples of fishes that can survive exposure to severe (>15 mm Hg PCO_2) hypercarbia, "air breathing" refers to whether there are examples of air breathing fish species, and "preferential pHi regulation" refers to whether complete pHi protection during severe pHe depression has been observed in any species. A dash "—" indicates no data exist for this group, and an "i.e." indicates indirect evidence exists for this category (see text for details). Phylogeny modified from Janvier, 2005. 172

Figure 7.3 The relationship between blood pH (pHe) and intracellular pH (pHi) of heart following recovery during exposure to short-term (3-96 h) hypercarbia (30 and 45 mm Hg PCO_2) in Pacific hagfish. Each point represents a single animal. Dotted lines represent 95% confidence intervals. Blood pH and heart pHi are significantly correlated ($p < 0.001$, $m = 0.29$, $r^2 = 0.71$) 174

Figure 7.4 The effect of short-term (96 h) hypercarbia (30 and 45 mm Hg PCO_2) on blood pH (pHe) and plasma [HCO_3^-] in Pacific hagfish as represented on a pH/HCO_3^-/CO_2 plot. Values are means ± s.e.m. (n = 6-8). Note plasma [HCO_3^-] are observed much greater than the 27-33 mmol l^{-1} threshold describe in teleosts (see Chapter 1 for more details). Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point represent exposure time. The dotted line indicates intrinsic buffer value of whole blood. Data and buffer values from Baker, Sardella, Rummer and Brauner, unpublished. 175

Figure 7.5 The effect of short-term (24-72 h) hypercarbia (11.5, 22.5 and 45 mm Hg PCO_2) on blood pH (pHe) and plasma HCO_3^- in *Amia calva* as represented on a pH/HCO_3^-/CO_2 plot. Values are means ± s.e.m. (n = 3). Note data points fall below the blood buffer line during early (3 h) exposure to hypercarbia, indicating the contributions of acid equivalents to the blood, presumably from the intracellular compartment (although not RBC) (see text for details). Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point

represent exposure time. The dotted line indicates the non-bicarbonate (i.e., intrinsic) buffer value of whole blood. Data and buffer values from Baker and Brauner, unpublished..... 176

LIST OF SYMBOLS AND ABBREVIATIONS

Hypercarbia classification:

ambient	0.2 mm Hg PCO_2
low	≤10 mm Hg PCO_2
moderate	>11 and <15 mm Hg PCO_2
severe	≥15 mm Hg PCO_2

β-NHE	β-adrenergically-activated sodium proton exchange
Δ	delta, change (e.g. ΔpH)
°C	degrees Celcius
AD	adrenaline
ADP_{free}	free cytosolic ADP
ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	adenylate cyclase and 3', 5' - cyclic monophosphate
Cl^-	chloride
CO_2	carbon dioxide
Cr	free creatine
CrP	phosphocreatine
DA	dorsal aorta
f_H	heart rate
f_T	tail beat frequency
f_V	ventilation frequency
F_V	maximum rate of force generation in ventricle

H^+	proton
Hb	haemoglobin
HCT	haematocrit
HCO_3^-	bicarbonate
K^+	potassium
KCl	potassium chloride
LN_2	liquid nitrogen
MCHC	mean cell haemoglobin concentration
mm Hg	millimeters of mercury, where 1 mm Hg is 1 torr or 0.1333 kPa
$\dot{M}_{O_2}$	oxygen consumption rate (mg O_2 kg^{-1} h^{-1})
MRC	mitochondrial rich cells
MS-222	tricaine methane sulphonate (anaesthetic)
N_2	nitrogen
Na^+	sodium
$NaHCO_3^-$	sodium bicarbonate
NHE	sodium proton exchanger
NKA	Na^+, K^+, ATPase
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid, disodium salt
O_2	oxygen
P_{in}	input pressure
P_{out}	output pressure
Pi	inorganic phosphate
PO	cardiac power output
PO_{max}	maximum cardiac power output

PO_2	partial pressure of O_2
PCO_2	partial pressure of CO_2
pHe	extracellular pH, blood pH
pHi	intracellular pH
PVC	pavement cells
Q	cardiac output
Q_{max}	maximum cardiac output
RBC	red blood cell
s.e.m.	standard error of the mean
V_S	cardiac stroke volume
VIU	Vancouver Island University
UBC	University of British Columbia

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DEDICATION

I dedicate this dissertation to my wife, Raegan Leigh Fitch, without whom this achievement would have been impossible.

1: GENERAL INTRODUCTION

1.1 Overview

Regulation of pH is central to survival in all vertebrates (Boron and De Weer, 1976; Heisler, 1986). In fishes, initial exposure to aquatic hypercarbia (elevated water PCO_2) induces a reduction in blood pH (pHe) and tissue intracellular pH (pHi) (i.e., a respiratory acidosis). The changes in intracellular pH (pHi) are qualitatively similar to, albeit smaller than, blood pH changes (Putnam and Roos, 1997; Brauner and Baker, 2009). Most fishes studied to date respond to low ($\leq$ 10 mm Hg PCO_2) or moderate (11-15 mm Hg) hypercarbia by slowly (over 24 to 48 h) compensating for the hypercarbia-induced acidosis by increasing net acid excretion, mainly at the gill, which drives pH recovery in both the blood and tissues (Heisler, 1999; Perry and Gilmour, 2006). This compensatory strategy, however, cannot recover blood pH (pHe) following transfer to severe ($\geq$ 15 mm Hg PCO_2) hypercarbia, likely due to limitations associated with branchial acid-base relevant ion transport (Rothe and Heisler, 1987; Wood et al., 1990; Heisler, 1999; Brauner and Baker, 2009). Thus, during severe hypercarbia, intracellular pH (pHi) remains depressed, and as the function of many cellular components, such as enzyme activity, is pH sensitive, the resultant respiratory acidosis may have severe consequences on cellular processes (Heisler, 1999; Putnam and Roos, 1997). Fishes that survive transfer to water CO_2 tensions greater than this apparent threshold to pHe compensation and CO_2 tolerance in fish of about 15 mm Hg PCO_2 must therefore have other compensatory strategies for coping with the induced intracellular acidosis.

Some fishes survive exposure to CO_2 tensions above the apparent threshold for days (15-45 mm Hg; e.g., Heisler, 1982; Crocker and Cech, 1998; McKenzie et al., 2003; Brauner et al., 2004; Hayashi et al., 2004). While mechanisms responsible for CO_2 tolerance remain to be

elucidated (Brauner and Baker, 2009), a few of these CO_2-tolerant species have demonstrated an exceptional capacity for pHi recovery despite sustained pHe depressions. This likely protects critical tissues by avoiding a prolonged tissue acidosis (Heisler, 1986; Brauner et al., 2004). This response, referred to as preferential pH regulation, might then support survival during episodes of aquatic hypercarbia and consequently preferential pHi regulation has been hypothesized to be associated with CO_2 tolerance in fishes. However, to date this response has only been observed in two facultative air-breathing fishes and so may instead be an adaptation to breathing air episodically. The white sturgeon, *Acipenser transmontanus,* has been shown to be exceptionally tolerant to hypercarbia, but is an exclusive water breather. Therefore white sturgeon were chosen as a model species in which to investigate the strategies associated with CO_2 tolerance.

In this thesis, I investigated whether the CO_2-tolerant white sturgeon exhibits preferential pHi regulation during aquatic hypercarbia. My aim was to determine whether preferential pHi regulation may be associated with CO_2 tolerance in sturgeon, and thus provide insight into strategies of CO_2 tolerance in fishes. The remainder of this chapter presents a background on the physiological response of fish to aquatic hypercarbia, challenges associated with severe hypercarbia, and a brief summary of what is known about CO_2-tolerant fishes, including the white sturgeon. This information will be followed by an outline of the thesis objectives and organization.

1.2 Acid-base regulation in fishes

Regulation of pH is central to survival in most vertebrates in both the cellular and extracellular compartments (Boron and De Weer, 1976, Heisler, 1986), as changes in pH can alter local charges on proteins and thus can affect protein function through, for example, modification of enzyme and membrane channel properties. These changes in turn can ultimately

affect cellular processes such as cell-to-cell signalling, volume regulation, and gene expression, but also whole animal performance (Putnam and Roos, 1997). All cells have some capacity to maintain intracellular pH homeostasis, but the degree to which they can defend cytoplasmic pH during an acid-loading event depends on the origin and severity of the acidosis (i.e., environmental, respiratory or metabolic) and the buffering capacity of the cell (Heisler, 1999; Putnam, 2001). In addition, active compensation of extracellular pH (i.e., blood pH) aids intracellular pH regulation and therefore is an important strategy for mediating an acidosis in many vertebrates (Truchet, 1987). In fishes, the specific mechanisms and general patterns of acid-base regulation have been the subject of many studies over the last several decades (e.g., Lloyd and White, 1967; Heisler, 1982; Claiborne and Heisler, 1986; Wood and LeMoigne, 1991; Goss et al., 1998). While much is known about pH regulatory strategies and mechanisms, a great deal remains to be discovered at the molecular, cellular and organismal levels.

This paucity of knowledge is especially evident with respect to acid-base regulation in fishes that exhibit a relatively high tolerance to elevated CO_2 and the associated acidosis. Many questions in comparative acid-base physiology remain to be answered including, for example, what is the physiological origin of the great variability in CO_2 tolerance among water breathing vertebrates? How do CO_2-tolerant fishes survive PCO_2 and pH perturbations known from research on other animal models (primarily mammalian) to be lethal? Is this variability the result of adaptation to selective pressures? These questions have been receiving even more attention lately, due to both technological advances which allow for greater accuracy and more detailed analysis of pH and PCO_2 of vertebrates *in vivo*, and renewed interest from scientific and public forums.

1.3 The importance of investigating aquatic hypercarbia

Environmental hypercarbia has been used as an experimental probe extensively by physiologists to investigate the mechanisms and patterns of acid-base regulation in fish, and, in particular, the role of the gill, intestine and kidney in organismal pH compensation. Consequently, a fair amount has been published about the responses of fish to environmental CO_2 tensions of 5-12 mm Hg, including hypothetical models describing cellular mechanisms of acid-base transport in ionoregulatory organs. Over the last 30 years, this scientific pursuit has also led to continued discoveries of CO_2-tolerant species and patterns of acid-base regulation in response to short-term (i.e., up to days) hypercarbia that represent deviations from generally accepted physiological responses of fishes.

Aquatic hypercarbia is not only an experimental tool, but also represents an acid-base challenge with great relevance both historically and presently. Aquatic hypercarbia has been observed in freshwater (e.g., seasonal ponds, Ultsch, 1996), brackish (e.g., estuaries) and marine (e.g., deep sea sites, Heisler, 1986; tide pools, Burggren and Roberts, 1991) ecosystems and may be quite severe. For example, in tropical fresh water systems, aquatic PCO_2 levels of up to 60 mm Hg have been observed (Heisler, 1982; Ultsch, 1996). Exposure to this level of hypercarbia would induce a 20-30 fold increase over resting arterial PCO_2 of fish in water equilibrated to atmospheric CO_2 tensions (i.e., during normocarbia). Contributing factors to CO_2 elevation can include thick surface vegetation, poor water mixing, thermostratification, high flora or fauna biomass, and anaerobic metabolism of micro-organisms (Heisler, 1999; Ultsch, 1996). Global environmental trends over the last 400 million years include many extended periods where these conditions may have existed for a significant proportion of the aquatic ecosystems on the planet. In part because of the above findings, a re-assessment of the importance of the role of CO_2 as a selective pressure during vertebrate evolution has been called for (Ultsch, 1996).

In addition to occurring naturally, hypercarbia can be induced through anthropogenic activities, and thus these activities represent new threats to fishes (Pörtner and Farrell, 2008). The relevance of aquatic hypercarbia, in particular as a result of global climate change and anthropogenic activity, has never been more clear than it is now. As a result of anthropogenic activities, globally projected increases in atmospheric CO_2 levels over the next several centuries are hypothesized to elevate surface water CO_2 levels 5-fold, which may reduce the pH of these waters by 0.7 pH units (Caldeira and Wickett, 2003). While this predicted level of hypercarbia is relatively low compared to the environmental levels described above, sequestering atmospheric CO_2 to deep ocean or geological sites through high pressure injection has been proposed as a means to prevent further increases in atmospheric CO_2. This procedure would have the potential to create environmental point sources of CO_2 that could result in CO_2 tensions greater than any naturally occurring levels, past or present, and consequently could create a severe challenge for aquatic organisms (Seibel and Walsh, 2001). Given the anthropogenic potential for generating such high levels of hypercarbia, there is renewed interest in assessing CO_2 tolerance and understanding the compensatory physiological responses in fish during exposure to high CO_2 levels (10-60 mm Hg, Hayashi et al., 2004; Ishimatsu et al., 2004; Kikkawa et al., 2004; Pörtner et al., 2004; Ishimatsu et al., 2004).

What follows is a description of how fish typically respond to hypercarbia, including an examination of what is known about pH compensation for the respiratory acidosis induced during short-term (up to 96 h) exposure to elevated CO_2 tensions.

1.4 Physiological responses of fishes to aquatic hypercarbia

Aquatic hypercarbia induces a rapid and general respiratory acidosis in fishes. This acid-base disturbance can be either minimized or compensated by the following mechanisms: a)

physicochemical buffering with intrinsic (i.e., non-bicarbonate) buffers, b) a change in ventilation to alter PCO_2 and thus pH, *via* the CO_2-HCO_3^- buffer system, or c) net transport of acid-base equivalents between the cell and the blood compartment and/or the blood compartment and the environment (see Evans et al., 2005). In general, the role of buffering is limited to minimizing early acid-base disturbance, but the buffer capacity in the blood and intracellular compartment is relatively small compared to the acidosis induced by small changes in PCO_2 (Heisler, 1986). Therefore, this mechanism cannot be heavily relied upon during a general acidosis. In addition, the combination of a high ventilatory requirement for O_2 uptake and a high CO_2 capacitance of water relative to O_2 results in internal PCO_2 levels and [HCO_3^-] being much lower in water-breathers than those in air-breathers (Desjours, 1988). Thus, adjustments in gill ventilation can only have moderate effects on acid-base regulation in water-breathing fish (Gilmour, 2001). Clearly, more research is required to understand the role of both intrinsic buffering and breathing on acid-base status in fish (See Gilmour, 2001; Perry and Gilmour, 2006). However, the current consensus is that net transport of acid-base equivalents is the predominant pathway for compensating for pH disturbances.

Studies on acid-base regulatory responses to aquatic hypercarbia in fishes are limited to a relatively small number of fishes, almost all of which are teleosts (e.g., rainbow trout, *Oncorhynchus mykiss*, Lloyd and White, 1967; Hyde and Perry, 1989; Wood and LeMoigne, 1991; Goss and Perry, 1994; Larsen and Jensen, 1997; Bernier and Randall, 1998; common carp, *Cyprinus carpio*, Claiborne and Heisler, 1984; brown bullhead, *Ictalurus nebulosus*, Goss et al., 1992; *Anguilla anguilla*, McKenzie et al., 2002; *Conger Conger*, Toews et al., 1983; *Fundulus heteroclites*, Edwards et al., 2005; cod, *Gadus morhua*, Larsen et al., 1997; Tench, *Tinca tinca*, Jensen and Weber, 1985; Japanese amberjack, *Seriola quinqueradiata*, Ishimatsu et al., 2004; the bastard halibut, *Paralichthys olivaceus*, Hayashi et al., 2004) or elasmobranchs (little skate, *Raja ocellata*, Graham et al., 1990; Wood et al., 1990; dogfish, *Scyliorhinus stellaris*, Heisler et al.,

1988; *Squalus acanthias*, Claiborne and Evans, 1992). The "typical" acid-base regulatory response in these fishes consists of a respiratory acidosis followed by pHe and pHi recovery over the following 24-96 h. This initial acidosis is rapid, as arterial PCO_2 equilibrates with water PCO_2 within minutes (Bernier and Randall, 1998), and pHe and pHi decrease as a function of both the newly-equilibrated CO_2 tension and the intrinsic buffer value of the respective compartment. As a visual aid, this acidification in the blood is represented on a $pH/HCO_3^-/CO_2$ plot for rainbow trout, *O. mykiss*, in Fig. 1.1 (between 0 and 24 h, data are taken from Larsen and Jensen, 1997). The dotted line on this and all subsequent $pH/HCO_3^-/CO_2$ plots throughout the thesis represents the intrinsic buffer capacity of the tissue in question, which in this case is whole blood. Tissues have greater intrinsic buffer capacity than the blood due mainly to higher intracellular protein concentrations, and so the initial intracellular acidosis is 30-70% less severe than that in the blood, yielding a $\Delta pHi/\Delta pHe$ of 0.3-0.7 that is both tissue and species specific (Fig. 1.2).

Following a respiratory acidosis, blood pH recovery is associated with branchial acid-base relevant ion transfer, which over hours to days (Larsen and Jensen, 1997), returns blood and tissue pH to normocapnic levels. This pHe recovery is accomplished through net acid excretion or net base accumulation, and drives pH along the respective PCO_2 isopleth during sustained hypercarbia. The compensatory response (with net H^+ flux represented by $[HCO_3^-]$) is shown for rainbow trout in Fig. 1.1 (between 2 and 24 h). The elevation in plasma $[HCO_3^-]$ in most fishes studied to date is matched by an equimolar decrease in plasma $[Cl^-]$ to maintain electroneutrality (Goss et al., 1998; Claiborne et al., 2002). While the specific transporters responsible for pH compensation remain largely unknown (Claiborne et al., 2002; Evans et al., 2005; Perry and Gilmour, 2006; Parks et al., 2009), there are many studies that provide indirect evidence through, for example, changes in mRNA expression patterns and protein levels of putative transporters in the gills [e.g., Na^+/H^+ exchangers (NHE) in marine species, V-type H^+-ATPases (V-ATPase)

coupled to apical membrane Na^+ channels (ENaC) in freshwater species, HCO_3^-/Cl^- exchange *via* transporters belonging to the SLC26 and 24 multi-gene families] in response to acid or base loading events (Claiborne et al., 2002; Evans et al., 2005; Perry and Gilmour, 2006). Further support for transport mechanisms comes from the experimentally observed morphological alterations of specific cell types in the gill epithelium (as described below) during exposure to hypercarbia and other acid-base disturbances (see Goss et al., 1998; Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005; Perry and Gilmour, 2006).

While pHe compensation for a respiratory acidosis can be initiated quickly and net H^+ efflux increased within 60 min (Wheatley et al., 1984; Edwards et al., 2005), a less rapid (hours to days) but extensive gill remodelling that occurs during hypercarbia is also thought to play a role in pHe recovery. In the gills of most teleosts studied to date, exposure to hypercarbia results in an increase in apical surface area of acid excreting cells through proliferation of microridges, and a concurrent decrease in the fractional surface area of base secreting cells through physical covering by adjacent cells (Goss et al., 1994; Goss et al., 1995; Goss et al., 1998). By altering the cell surface area exposed to the environment, and thus sites for ion transport in the respective cell types, these morphological changes in the gill may potentially aid in either increasing acid efflux or limiting base efflux during exposure to hypercarbia (see Perry, 1997; Goss et al., 1998; Evans et al., 2005; Perry and Gilmour, 2006).

Because the gills have traditionally been thought to account for approximately 90% or more of the net acid-base relevant ion transport in fish during compensation for an acid-base disturbance in freshwater and seawater (Cameron, 1989), this organ has been investigated most intensively. Interest in the role of the kidney and intestine in whole animal acid-base regulation has increased of late (see Evans et al., 2005, Perry and Gilmour, 2006 for reviews), and it appears they play a greater role than previously thought. Still, quantitatively these organs remain

secondary to the role of the gills. Consequently, this thesis has been limited to examination of the gill as an organ associated with net acid excretion during the correction of an acidosis.

The responses described above (net H^+ excretion and gill remodelling) are effective in driving pH recovery during hypercarbia, and, albeit with modification, represent the paradigm for acid-base regulation during hypercarbia in water-breathing fish (Evans et al., 2005; Perry and Gilmour, 2006). However, there are limitations to pHe compensation during hypercarbia which is the focus of the next section.

1.5 Limitations to pHe compensation during aquatic hypercarbia

As hypercapnia and the resulting acidosis increase in severity, the capacity of a fish to achieve complete pHe compensation through net HCO_3^-/Cl^- exchange becomes reduced. By extrapolation along the respective PCO_2 isopleth in Fig. 1.3 (see figure caption for further details), it is clear that a fish exposed to a PCO_2 of 30-50 mm Hg, would have insufficient Cl^- in the plasma to match the HCO_3^- accumulation necessary (i.e., > 100-120 mmol l^{-1}) for complete pH compensation for the respiratory acidosis induced. Thus, ultimately net HCO_3^-/Cl^- exchange has limits based on Cl^- availability. However, net HCO_3^- accumulation plateaus at HCO_3^- and PCO_2 levels much lower than this absolute limit (Fig. 1.3). The observation of this limit to pHe compensation has been referred to as "the bicarbonate concentration threshold" (Heisler, 1986; Heisler, 1999), and has significant implications with regards to CO_2 tolerance.

This threshold was described almost 30 years ago, yet the source of the limitation remains unresolved. However, some water characteristics (e.g., water pH, salinity and hardness) are believed to be related to the rate and magnitude of pHe compensation. Water with low pH will have a lower $[HCO_3^-]$ available for uptake (Heisler, 1986; Heisler, 1999), but in addition, environmental pH can affect the rate of net H^+ efflux in branchial tissue. For example, the

relative activity of V-ATPase, which may play an important role in pH compensation during hypercapnia (Heisler, 1999; Perry and Gilmour, 2006), decreases as water pH decreases in rainbow trout (Lin and Randall, 1990). Also, in both dogfish and carp, pH recovery was severely compromised in acidified water (Heisler, 1999). The effects of water hardness (as measured by $CaCO_3$) and salinity through increased water buffer capacity, appears to increase the rate and proportion of pH recovery in fish exposed to short term hypercapnia (Heisler, 1988; Heisler, 1999). However, higher salinity may also exert its mediating influence through availability of counter ions (Iwama and Heisler, 1991), and increasing Ca^{2+} has been hypothesized to reduce passive HCO_3^- loss through ion-permeable cellular junctions in the gills of some fish (Heisler, 1999), although strong experimental support for these hypotheses is lacking. The observation that the rate and degree of pH compensation is influenced by water characteristics might have interesting implications for the origin of alternate strategies for surviving hypercarbic challenges (Brauner and Baker, 2009).

Because of this limitation to pHe compensation, net HCO_3^- uptake in exchange for Cl^- is constrained as a viable short-term strategy to CO_2 levels below 10-15 mm Hg (see Fig. 1.3) (for the few exceptions, see Heisler, 1986; Heisler, 1999; Hayashi et al., 2005; Perry et al., 2010). Not surprisingly, some species of fish do not survive CO_2 levels above this. For example, when exposed to environmental PCO_2 of 37.5 mm Hg, the Japanese amberjack (*S. quinqueradiata*) did not survive 8 h. At the same tension, the bastard halibut (*P. olivaceus*) was unable to survive 48 h, and 17% had died by 8 h (Ishimatsu et al., 2004). Rainbow trout directly transferred to water equilibrated with a PCO_2 of 30 mm Hg did not survive even 3 h (D. Baker, personal observation). The sensitivity of these species to hypercarbia is likely associated with exceeding the capacity to compensate for the respiratory acidosis. Despite the proposed limit to pHe compensation, a few fish species tolerate CO_2 tensions well beyond this. What is known about

acid-base regulatory responses associated with high CO_2 tolerance is discussed in the following section.

1.6 CO_2-tolerant fishes and the challenge of severe hypercarbia

As mentioned above, active alteration of pHe aids in acid-base regulation and therefore is an important strategy for compensating an acidosis in many vertebrates (Truchot, 1987). In tissue from fishes studied to date, cellular pHi changes are qualitatively similar but quantitatively smaller compared to pHe during a respiratory acidosis (Rothe and Heisler, 1987; Wood et al., 1990; Wood and LeMoigne, 1991). The degree of acidosis experienced intracellularly is less that extracellularly because of the substantially higher intracellular intrinsic (i.e., non-bicarbonate) buffer capacity, which is mainly due to higher intracellular protein concentration. This relationship between pHe and pHi is the case for red blood cells, liver, and muscle in rainbow trout *in vivo* (although not the gill) (Wood and LeMoigne, 1991; see Fig. 1.2). Examination of isolated hepatocytes from rainbow trout supported this conclusion, as, when exposed to either 7.5 mm Hg PCO_2 or isocapnic acidosis, hepatocytes exhibited a $\Delta pHi/\Delta pHe$ of 0.51 (Walsh et al., 1988). In primary hepatocyte isolations from the Antarctic fish (*Pachycara brachycephalum*), depression of pHe through HCl addition at constant gas tensions (either atmospheric or 7.5 mm Hg), resulted in a depression in pHi, with a slope of $\Delta pHi/\Delta pHe$ of 0.4 after 50 min of incubation (Langenbuch and Pörtner, 2003). In skate exposed to hypercarbia *in vivo*, a depression of pHe resulted in a reduction of pHi in red blood cell, heart, brain, and muscle consistent with that observed in trout, although brain and heart tissue exhibited slightly more rapid pHi recovery (Fig. 1.2, data from Wood et al., 1990). This relationship between pHe and pHi implies that fish exposed to CO_2 levels beyond the limit for complete pHe recovery will

exhibit a persistent intracellular acidosis, although *in vivo* experimental support for this conclusion is limited (dogfish, Holeton and Heisler, 1983; carp, Claiborne and Heisler, 1986).

Few studies have investigated the relationship between pHe and pHi in CO_2-tolerant fish, especially at more severe CO_2 tensions. Even so, some fish defend pHi during large reductions in pHe. Prior to this thesis, only two facultative air breathers, the armoured catfish, *Pterygoplichthys pardalis*, (Brauner et al., 2004) and the marbled swamp eel, *Synbranchus marmoratus*, (Heisler, 1982) have been observed to regulate tissue pHi but not pHe during severe hypercarbia, and this strategy for pHi protection has been referred to as "preferential pHi regulation" (Brauner et al., 2004; Baker et al., 2009a; Brauner and Baker, 2009). Both of these species protect heart and white muscle pHi during PCO_2 increases of 20 mm Hg and greater during hypercarbia or hypoxia-induced air breathing. A number of water breathing species also exhibit CO_2 tolerance well beyond the capacity for pHe compensation (e.g., European eel, *A. anguilla*, McKenzie et al., 2003; bowfin, *Amia calva*, Pacific hagfish, *Eptatretus stoutii*, Brauner and Baker, 2009; carp, Claiborne and Heisler, 1984). These fishes must tolerate or protect against the intracellular acidosis associated with severe hypercarbia, but currently how these fishes survive is unknown.

Another fish known to be CO_2 tolerant is the white sturgeon, *Acipenser transmontanus* (Crocker and Cech, 1998; Crocker et al. 2000). I have chosen to use the white sturgeon to examine CO_2 tolerance in fishes, and the rationale for this choice is the focus of the next section.

1.7 White sturgeon as a representative species for CO_2-tolerant fishes

Anecdotally, the hypercarbia tolerance of sturgeons has been known for decades, mainly because of observations from aquaculture settings, where high density holding practices can result in extremely high aquatic water CO_2 levels (Sowerbutts and Forster, 1981; Colt and

Orwics, 1991). White sturgeon, *A. transmontanus* are highly tolerant of aquatic hypercarbia (Crocker and Cech, 1998; Crocker et al., 2000; Crocker and Cech, 2002), and can survive hypercarbia of 30 mm Hg PCO_2 for days, despite an extended acidosis in arterial blood (Crocker and Cech, 1998). During this exposure, net bicarbonate accumulation (and associated Cl^- loss) was almost absent and pHe compensation minimal (Crocker and Cech, 1998). Whether white sturgeon are capable of preferential pHi regulation during hypercarbia is unknown. All examples of preferential pHi regulation in fishes prior to the research described in this thesis have been in obligate or facultative air-breathers. Thus, should white sturgeon exhibit preferential pHi regulation during hypercarbia, they will represent both the most basal vertebrate and only exclusively water-breathing fish confirmed to have a capacity for exceptional tissue pHi protection (i.e., preferential pHi regulation), and this could be the basis for their high CO_2 tolerance.

There are a number of other reasons that sturgeon are well suited for examining hypercarbia tolerance. For example, sturgeons in general exhibit a minimal stress response to handling and other stressors, and they recover to resting levels quickly (e.g., cortisol, lactate; Barton et al., 2000; Baker et al., 2005a; Baker et al., 2005b). Thus, stress-related effects due to experimental protocols (e.g., handling, anaesthetic effects) may be less in sturgeons than those in more commonly investigated fishes (e.g., rainbow trout), and so confound less interpretation of hypercarbia-related responses. In addition, sturgeon haemoglobins do not exhibit a loss of oxygen saturation binding capacity (i.e., Root effect) at physiologically relevant RBC pHi (Regan and Brauner, 2010), and so, unlike in most teleosts, aquatic hypercarbia does not induce hypoxemia. This may also reduce confounding (i.e., hypoxemia-related) responses associated with hypercarbia exposure. Finally, white sturgeon in particular have been the subject of more research focussed on CO_2-related acid-base physiology than all but the most commonly used experimental fish models (e.g., trout and goldfish), probably due to the great interest of the

aquaculture industry in maximizing production of caviar (i.e., sturgeon roe) (Van Eenennaam et al., 2005), although the endangered status of sturgeons worldwide (Auer, 2005) may have also played a role. Consequently, the body of literature upon which to draw is not as limited as for most species currently known to be CO_2 tolerant.

The sturgeons represent an ancient chondrostean family of fishes over 250 million years old, and have enormous value for studying vertebrate evolution, including physiological adaptations to the environment (Cech and Doroshov, 2004). Consequently, research on acid-base physiology in sturgeons may not only address important questions about CO_2 tolerance in this resilient species, but could also potentially provide insight into the evolution of CO_2 tolerance in fishes.

1.8 Thesis objectives and outline

The primary objective of this thesis was to investigate the physiological basis for the exceptional CO_2 tolerance of white sturgeon. This required an approach integrating responses from the organismal, tissue and biochemical levels. The general hypothesis tested in this thesis was that survival of white sturgeon during short-term aquatic hypercarbia is associated with tight regulation of pHi in critical tissues. In subsequent chapters, 5 manuscripts will be presented that investigated aspects of this general hypothesis using white sturgeon as a representative species of CO_2-tolerant fishes.

Chapter 2: A validation of pHi measurements in fish exposed to aquatic hypercarbia: The effect of duration of tissue storage and efficacy of the metabolic inhibitor tissue homogenate method

Accurately assessing acid-base physiological response of white sturgeon to aquatic hypercarbia depended heavily on the accuracy of traditional methods for measuring pHi and estimating intrinsic buffering. Before a thorough examination of other aspects of preferential pHi regulation could be approached, the sensitivity of the method used for pHi measurement had to be addressed with regards to its use on tissues exposed to high CO_2 tensions. This chapter assessed the effect of tissue storage duration and CO_2 exposure level on the accuracy of the metabolic inhibitor method (MITH) for measuring pHi, using frozen RBC pellets as a representative of frozen tissues. As much of the work in this thesis relies on interpreting changes in pHi and estimating the contribution of active organismal and cellular acid/base relevant transport, the results of this chapter addressed possible methodological criticisms, and represented a critical first step in evaluating the importance of the role of preferential pHi regulation during aquatic hypercarbia.

Chapter 3: Is pHi preferentially regulated in white sturgeon during the pHe depression associated with aquatic hypercarbia?

Chapter 3 consists of a characterization of the physiological responses of the CO_2-tolerant white sturgeon to aquatic hypercarbia, to determine whether CO_2 tolerance was associated with an increased pH regulatory capacity (pHe or pHi) to protect tissues from the associated acidosis. In addition, physiological responses of white sturgeon were compared to those of other teleosts published in the literature, particularly with respect to the changes associated with pHe compensation. The findings from this chapter provided the foundation for the development of the rest of the thesis.

Chapter 4: What are the metabolic effects of aquatic hypercarbia over a range of CO_2 tensions in white sturgeon?

In Chapter 4, I examined changes in metabolism associated with aquatic hypercarbia over a range CO_2 tensions within and beyond those at which white sturgeon can endure for long periods (i.e., days). After observing survival, I investigated how whole animal oxygen consumption rate changed over this range of CO_2 tensions. Finally, I measured a suite of metabolically relevant organismal and biochemical parameters to gain insight into the metabolic costs associated with hypercarbia tolerance.

Chapter 5: What are the *in vivo* pHi and metabolic responses of white sturgeon during the initial exposure ($\leq$ 90 min) to severe aquatic hypercarbia, as determined by ^{31}P-NMR?

I used recent technological advances to assess *in vivo* changes in pHi and cellular metabolites in real-time in the heart of white sturgeon during the first 90 min of exposure to severe (45 mm Hg PCO_2) aquatic hypercarbia. Specifically, nuclear magnetic resonance (NMR) was used to collect ^{31}P spectra from white sturgeon hearts *in vivo*, and changes in pHi and relative concentrations of CrP and ATP were determined simultaneously. Finally, the values obtained using NMR were validated through comparison with values obtained from excised tissues using conventional techniques, similar to those methods used in other sections of the thesis.

Chapter 6: What is the effect of elevated CO_2 on white sturgeon maximum cardiac performance as assessed on an isolated heart *in situ*? Will adrenergic stimulation alter this performance?

In this chapter, I investigated whether the exceptional CO_2 tolerance was extended to maximum performance of a life-support organ normally regarded as being acidosis intolerant, the heart. I used an *in situ* heart preparation to assess how cardiac performance during elevated CO_2 in the blood (hypercapnia) might be protected during severe hypercapnia (45 mm Hg PCO_2). Maximum cardiac performance was assessed in hearts during perfusion with CO_2 equilibrated salines. As elevated adrenaline concentrations have been observed during hypercarbia, hearts were assessed again with the addition of saturating levels of this stress hormone. This work was undertaken to determine whether tissue pHi compensation might be accomplished at a cost to organ performance (e.g., maximum cardiac work).

Chapter 7 of this thesis is a general discussion, where the ideas generated and conclusions drawn from the aforementioned chapters are summarized, and then placed within the broader context of the evolution of acid-base physiology in water breathing vertebrates. The final section of this chapter suggests important future directions that could be examined as an extension of this work.

1.9 Figures

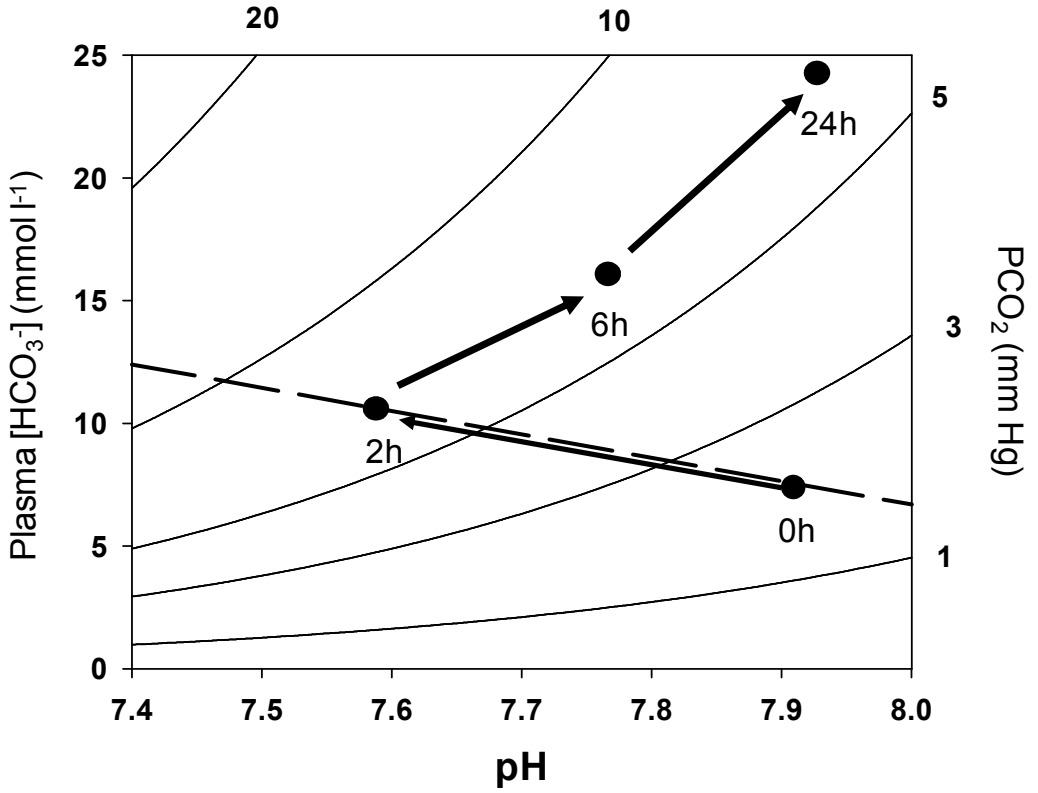


Figure 1.1 The effect of short-term (24 h) of low hypercarbia (7 mm Hg PCO_2) on blood pH and plasma HCO_3^- in rainbow trout as represented on a pH/HCO_3^-/CO_2 plot. Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point represent exposure time; arrows indicate temporal pattern of change in blood pH and plasma HCO_3^-. The dotted line indicates the intrinsic (i.e., non-bicarbonate) buffer value of whole blood as reported by Wood and LeMoigne (1991) (see text for further details). Data from Larsen and Jensen (1997).

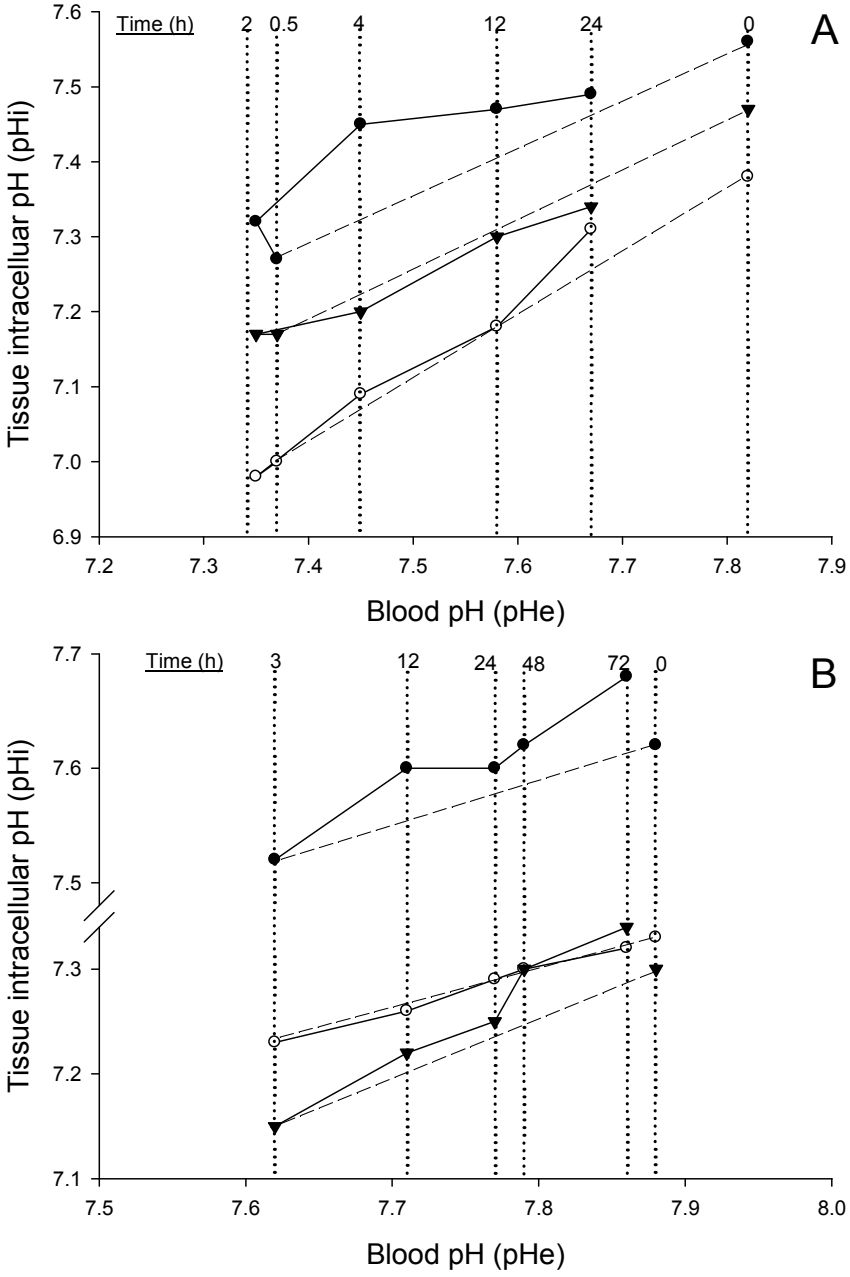


Figure 1.2 The relationship between the pH of blood (pHe) and intracellular pH (pHi) of red blood cells (open circles), brain (filled circles), and white muscle (inverted triangle) during exposure to short term low hypercarbia in (A) little skate, *R. ocellata*, and (B) hyperoxia-induced hypercarbia in rainbow trout, *O. mykiss*. Time course (h) is indicated by numbers located directly above vertically oriented groupings. Note reversal of early time points (0.5 and 2 h) in panel A. While pHi can recover more rapidly than pHe in tissues such as the brain and heart of skate, in all tissues presented here, pHi remains depressed if pHe does not recover. Mean values and s.e.m. bars approximated from Wood et al. (1990) for skate and Wood and LeMoigne (1991) for trout.

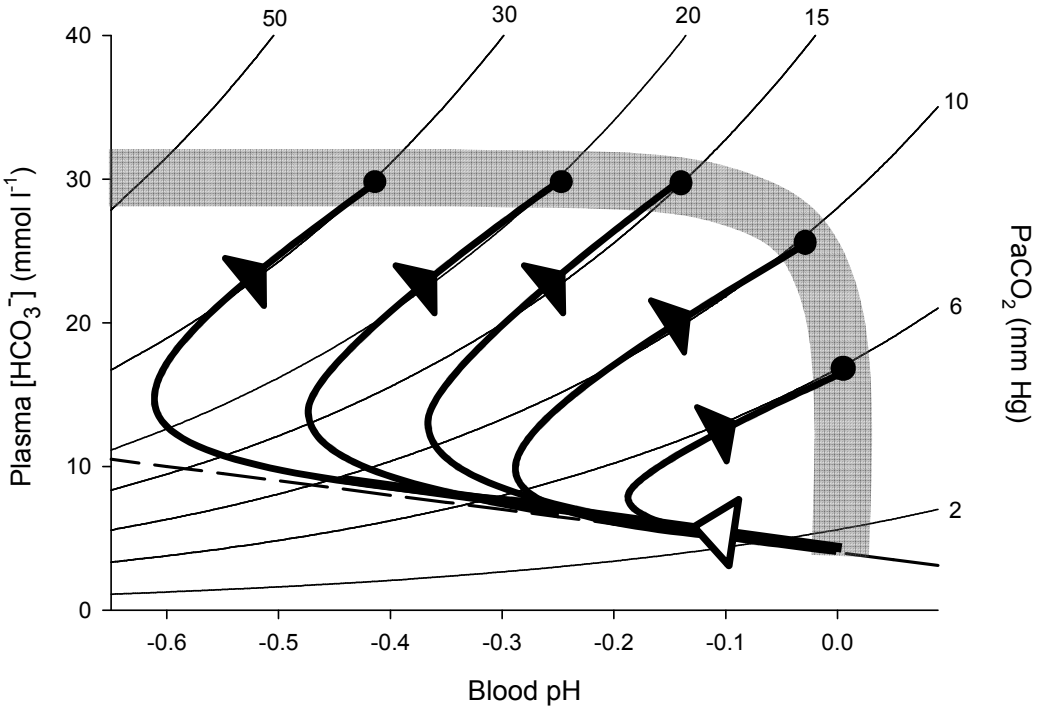


Figure 1.3 A theoretical representation of the "typical" temporal response to short-term (less than 96 h) hypercarbia in fish. Upon transfer from normocarbia to hypercarbia, blood pH rapidly falls along the non-bicarbonate buffer line of the blood as indicated by black open arrowhead, and pH recovers along a given PCO_2 isopleth through a net increase in HCO_3^- in exchange for Cl^- as indicated by black filled arrowheads. Black filled circles represent final pHe values that would be achieved based upon apparent limits to net HCO_3^- accumulation within 24-96 h of exposure to hypercarbia (see text for further details). Shaded bar indicates maximal pH compensation limited by the "bicarbonate concentration threshold", as most fish do not increase plasma HCO_3^- beyond 25-35 mmol l^{-1} (modified from Heisler 1986, 1999). Thus, compensation for a respiratory acidosis (within 48-96h) during exposure to hypercarbia is incomplete above a PCO_2 of 10-15 mm Hg in most water-breathing fishes. Note that CO_2 isopleths are dependent on both temperature and osmolarity, and that the isopleths represented here are plotted for clarity purposes only.

2: A VALIDATION OF INTRACELLULAR pH MEASUREMENTS IN FISH EXPOSED TO HYPERCARBIA: THE EFFECT OF TISSUE STORAGE DURATION AND EFFICACY OF THE METABOLIC INHIBITOR TISSUE HOMOGENATE METHOD

2.1 Synopsis

Using red blood cells from white sturgeon (*Acipenser transmontanus*) as a representative tissue, here I assessed the accuracy of the metabolic inhibitor tissue homogenate (MITH) method of measuring intracellular pH. RBC pHi was measured following equilibration of RBC to a range of CO_2 tensions, including normocapnic (3.75 mm Hg PCO_2) and very high levels (≤ 75 mm Hg PCO_2). In addition, the effect of tissue storage duration on RBC pHi from fishes exposed to high PCO_2 was investigated as a possible source of error due to diffusive loss of CO_2. Only minor effects of long term (90 days) storage were observed and there was no significant effect of storage in liquid nitrogen for up to 30 days. More importantly, pHi measured using the MITH method returned values similar to those obtained by the previously validated freeze-and-thaw method (FAT) in red blood cells exposed to hypercarbia up to 75 mm Hg PCO_2. Consequently, the MITH method is suitably accurate for determination of pHi from excised tissues in fishes exposed to aquatic hypercarbia.

2.2 Introduction

Acid base regulation is critical for proper function of cellular processes, such as energy production, metabolism and contractile force generation (Putnam, 2001). Consequently, pH is tightly controlled under normal conditions (see review in Putnam and Roos, 1997). Interest in the

effects of the respiratory acidosis associated with elevated environmental CO_2 (hypercarbia) on aquatic animal physiology has recently been stimulated due in part to a growing concern about anthropogenic carbon dioxide production (Chapter 1; Hayashi et al., 2004; Pörtner et al., 2004; Brauner and Baker, 2009). Current projections of atmospheric CO_2 levels suggest that surface waters may see as much as a five-fold increase in ambient levels over the next several centuries (Caldera and Wickett, 2003). In response to this, sequestration and relocation of atmospheric CO_2 to deep ocean sites via high pressure injection has been proposed as a means to reduce the rate of CO_2 accumulation (Seibel and Walsh, 2001), a practice that would create point sources of CO_2 with no historical precedent. While the effect of acute CO_2 exposure on gas exchange and acid-base regulation in fish is well studied at low CO_2 tensions (< 10 mm Hg PCO_2, for examples, see Claiborne and Heisler, 1986; Heisler et al., 1988; Graham et al., 1990, Wood et al., 1990; Wood and LeMoigne, 1991; Goss and Perry, 1993), there are relatively few studies documenting the extent of CO_2 tolerance and its physiological effects in fish (Chapter 1; Brauner and Baker, 2009). As aquatic hypercarbia, both moderate (below 15 mm Hg PCO_2) and severe (up to 60 mm Hg PCO_2), is now believed to be more common in freshwater and saline environments than previously thought based on field measurements (e.g., tropical freshwater systems, Heisler, 1982; Ultsch, 1996), a number of studies have been published recently to investigate hypercarbia tolerance (for example, Hayashi et al., 2004; Ishimatsu et al., 2004; Pörtner et al., 2004; Ishimatsu et al., 2005; Brauner and Baker, 2009).

Gills and cell membranes in fish are highly permeable to CO_2, and thus, the resultant acidosis induced by aquatic hypercarbia is experienced both extra- and intracellularly. Regulation of pH in these compartments is probably important for tolerance and survival of severe hypercarbia (see Chapter 1; Brauner and Baker, 2008). While measurement of blood pH (pHe) is relatively simple as it can be directly measured using a pH electrode, obtaining accurate values for intracellular pH (pHi) is more challenging. The few techniques used to measure pHi

from tissues can involve great expense (e.g., ^{31}P-NMR), require specialized equipment or permits, are highly derivative, and lack temporal resolution [e.g., the use of the pH-dependent distribution of radio-labelled 5,5-dimethyl-2,4-oxazolidinedione (DMO), Pörtner et al., 1990]. Another technique, the freeze-thaw method (Zeidler and Kim, 1977, referred to hereafter as FAT) has only been used effectively with isolated red blood cells. The metabolic inhibitor tissue homogenate method (abbreviated as MITH), which requires pulverizing a tissue sample to a fine powder while cooled by liquid nitrogen, and then suspending the resultant homogenate in a chilled metabolic inhibitory cocktail, was described and evaluated by Pörtner et al. (1990). This method was verified at normal PCO_2 levels, and found to be effective for measuring pHi in excised muscle tissue from a number of animal species sampled [worms (*Sipunculus nudus* L.), squid (*Illex illecebrous* L.), rainbow trout (*Oncorhynchus mykiss* Walbaum) and toad (*Bufo marinus*)] (Pörtner et al., 1990). This MITH method provides improvements over earlier methods (Costill et al., 1982; Spriet et al., 1986), and has been validated in relation to the DMO technique (e.g., Pörtner, 1987, Milligan and Wood, 1985). Furthermore, it is inexpensive, involves a relatively simple protocol, requires little equipment (a pH electrode and meter), and can be used on any tissue that can be homogenized. It has subsequently been used in a number of studies focussed on examining changes in tissue pH as a result of temperature (e.g., Pörtner et al., 2004), hypoxia (e.g., Jibb and Richards, 2008), exercise (e.g., Richards et al., 2002) and hypercarbia (e.g., Brauner et al., 2004).

While hypercarbia can induce an acidosis *in vivo*, once the tissue has been excised and removed from the high CO_2 environment (i.e., the animal), there is a potential for diffusive CO_2 loss to the atmosphere (i.e., air), where levels are very low (0.2 mm Hg PCO_2). Continuous CO_2 loss would lead to bicarbonate dehydration and further alkalization of the sample prior to measurement. Clearly, as CO_2 levels are experimentally increased, the diffusive gradient between excised tissue and atmosphere becomes greater, and so too will the absolute rate of CO_2

loss and potential for pHi measurement error. While these errors may be limited by excising and freezing tissue rapidly using liquid nitrogen (LN_2), the rate of diffusive CO_2 loss is greatly dependent on the surface area of the tissue sample, and the effect of this loss is related to the surface area to volume ratio. A tissue stored in an environment with a significant CO_2 gradient (i.e., CO_2-free environment, LN_2) and pulverized to a fine powder with an extremely high surface area to volume ratio may be especially prone to CO_2 loss. Thus, even with careful sample storage and preparation using MITH, CO_2 loss, especially from severely hypercapnic tissues, could potentially result in erroneous pHi measurements from hypercarbic tissues.

While this potential pHi measurement error through diffusive CO_2 loss could occur in tissues from any animal exposed to hypercarbia, pHi in fish tissues may be affected to a greater extent than air breathers for two reasons. First, fish tissues have very low normocarbic PCO_2 *in vivo* (2-4 mm Hg PCO_2), and environmentally relevant challenges may increase these levels twofold to twentyfold or more (Ultsch, 1996). Second, tissues of water breathing animals typically have been thought to have less intrinsic buffering than those of air breathers (by factors of 1.5 to 4, Heisler, 1999), although supporting evidence is lacking. McKenzie et al. (2003) noted that, in tissues excised from eels (*Anguilla anguilla*) exposed to hypercarbia, pHi was altered if, during preparation, tissue homogenates had access to air during centrifugation; this effect was assumed to be through diffusive CO_2 loss. As accurate pHi measurements are essential for understanding CO_2 tolerance and effects during experimental exposure, the degree to which these potential sources of error (i.e., storage duration and sample preparation) may affect pHi measurements under conditions of hypercarbia needs to be characterized.

Consequently, the objectives of this research were 1) to assess the effect of tissue storage duration on pHi in tissues from white sturgeon exposed to hypercarbia, and 2) to assess the accuracy of the MITH method for determining pHi in tissues exposed to a range of CO_2 levels, including very severe hypercarbia. To address the first objective, red blood cell (RBC) pHi of

white sturgeon exposed to hypercarbia (30 mm Hg PCO_2) was measured immediately after sampling, and then following storage of the frozen RBC pellet in LN_2 for 1, 7, 30 and 90 days or in an ultra cold (i.e., -80°C) freezer for 90 days. To address the second objective, whole blood from sturgeon was equilibrated in tonometers at 3.75, 7.5, 15, 30, 45,and 75 mm Hg PCO_2, respectively, and RBC pHi was measured by both the MITH and FAT methods. The FAT method has been verified as accurate in mammalian red blood cells, in which resting CO_2 levels are much higher (e.g., ~40 mm Hg PCO_2) than those in fish tissues, and so was used to validate the MITH method.

2.3 Materials and methods

2.3.1 Animals and rearing conditions

All experiments were performed in the Department of Zoology, UBC, Vancouver, B.C., Canada, in spring of 2008. White sturgeon (1.5-3 kg; 4 year old), progeny of Fraser River brood stock located at Vancouver Island University, Nanaimo, B.C., Canada, were held in aerated, flow through outdoor tanks (ambient light; T = 10-13°C), PO_2 > 130 mm Hg, PCO_2 < 0.2 mm Hg, water flow rate = 5 l min^{-1}, fish density < 25 kg m^{-3}).

2.3.2 Series 1: The effect of storage duration and method on pHi

For Series 1 (the effect of tissue storage duration on intracellular pH), white sturgeon were held in Plexiglass boxes provided with recirculating water that was equilibrated at 30 mm Hg PCO_2 generated using a Cameron gas mixing flow controller, verified by a thermostated Radiometer PCO_2 electrode (E5036) displayed on a Radiometer PHM 73. After 48 h, each box

was isolated, and an anaesthetic was added to the water directly (final concentration of MS-222, 0.3 g l^{-1}, buffered with $NaHCO_3$). Following cessation of ventilation (less than 3 min), animals were transferred to a surgery table, and blood was quickly drawn *via* caudal puncture into a heparinized syringe (10 ml, 23G1 needle), transferred to one of six 1.5 ml centrifuge tubes, and placed immediately on ice. Centrifuge tube lids were punctured by 16G needle prior to use to avoid trapping LN_2 during thawing. Fish were then moved to a recovery tank, and gills were flushed with oxygenated water. All animals began ventilating within 20 minutes of this treatment. Whole blood was centrifuged (3 min at 10,000 rpm) and the separated plasma and white blood cell layer was removed by pipette. The remaining packed red blood cell pellet was immediately frozen in LN_2 for 1, 7, 30, or 90 days or initially frozen in LN_2 and maintained in an ultracold (i.e., -80°C) freezer for 90 days.

2.3.3 Series 2: The effect of CO_2 tension on pHi measurements

For Series 2 (to assess the accuracy of the MITH method for determining intracellular pH in hypercarbic tissues), white sturgeon were transferred by net to an anaesthetic bath (final concentration of MS-222, 0.3 g l^{-1}, buffered to pH = 7.0 with $NaHCO_3$). Caudally sampled blood (as described above) was transferred to an Eschweiller thermostated (13°C) glass tonometer (4 ml), and allowed to equilibrate for 1 h at 3.75, 7.5, 15, 30, 45, or 75 mm Hg PCO_2) generated using a Wösthoff DIGAMIX 6KM 422 gas mixing pump (n = 4 for each CO_2 level). After equilibration, three aliquots of blood were drawn from the tonometer by syringe, and gently expelled into one of a 0.5, 1.5 or 2 ml centrifuge tube, and placed on ice. The smallest aliquot (0.5 ml) was immediately analyzed for whole blood pH (pHe). In the two remaining aliquots, RBC's were separated from plasma as described above and frozen in LN_2 prior to measurement of pHi using either the FAT or MITH method.

2.3.4 Intracellular pH measurements

RBC pHi was measured *via* the FAT method (described in Zeidler and Kim, 1977) and the MITH method (according to Pörtner et al., 1990). For the FAT method, each frozen RBC pellet in a 1.5 ml centrifuge tube were placed on ice until thawed, and returned to LN_2 until frozen, and this was repeated three times in quick succession. pH was then measured in the resulting homogenate. For the MITH method, the red blood cell pellet was pulverized with a LN_2-cooled mortar and pestle, and the resultant homogenate transferred *via* a pre-cooled metal scoop to a pre-cooled centrifuge tube under atmospheric conditions (i.e., low PCO_2). An aliquot of chilled (2°C) metabolic inhibitor solution (150 m mol^{-1} KCl and 8 mmol l^{-1} NTA) was then added to the homogenate. This tissue suspension was briefly stirred with a pre-cooled needle, and placed on ice for approximately 10 min. The pH of the mixture was then measured. All pH measurements were made in triplicate using a Radiometer microcapillary electrode (G299A) thermostated (13°C) in a Radiometer BMS 3 MK-2 system and displayed on a Radiometer PHM 73.

2.3.5 Statistical analyses

Statistical analysis was performed as follows: in Series 1, one-way analysis of variance (one-way ANOVA, α=0.05) was used to detect differences between CO_2 exposed samples over storage time. If differences were detected, Dunnett's post-hoc test was used to determine differences between stored samples and those measured immediately (i.e., no storage time). In Series 2, to determine whether there was a significant correlation between pHe and pHi as measured by either the FAT or MITH methods, a Student's t-test on the data set for each method

was performed (m = 0). The slopes and elevations between the two lines were tested for differences using a Student's t-test ($m_1 = m_2$; Zahr, 1984). Finally, the correlation between pHi values obtained for each sample by the two methods was compared to a slope of 1 using a Student's t-test (Zahr, 1984). Mean values for Series 1 are presented with s.e.m. bars (see figure captions for more details)

2.4 Results

2.4.1 Series 1: The effect of storage duration and method on pHi

RBC pHi of normocarbic white sturgeon was 7.26 ± 0.04. Following 48 h of exposure to 30 mm Hg PCO_2), RBC pHi of white sturgeon fell to 6.81 ± 0.01, as measured immediately after sampling. There was no significant change in RBC pHi following storage in liquid N_2 for up to 30 days. However, after 90 days of storage in LN_2, there was a small but statistically significant increase (~0.023 pH units, one-way ANOVA, $p < 0.05$; Fig. 2.1) in RBC pHi. A significant increase (~0.027 pH units, one-way ANOVAp < 0.05; Fig. 2.1) was also observed in samples that had been immediately transferred to an ultra cold (-80°C) freezer after initial freezing in LN_2 and subsequently stored for 90 days.

2.4.2 Series 2: The effect of CO_2 tension on pHi measurements

In blood equilibrated *in vitro*, blood pH (pHe) fell from 7.85 ± 0.02 at 3.75 mm Hg PCO_2) to 6.88 ± 0.01 at 75 mm Hg PCO_2) in a pattern consistent with that observed *in vivo* (Crocker and Cech, 1998; Brauner and Baker, 2009). There was no sign of cell lysis, even at the highest CO_2 treatment (1 h at 75 mm Hg PCO_2): plasma remained clear and colourless, and

haematocrit (HCT) before and after experimental protocol was similar (HCT: 31% vs. 29.5%). Consistent with the observed reduction in pHe, there was a reduction in RBC pHi from 7.28 ± 0.03 at 3.75 mm Hg PCO_2 to 6.67 ± 0.01 at 75 mm Hg PCO_2 as measured by the FAT method. When pHe was regressed against pHi (Fig. 2.2), a significant and positive correlation was observed (Student's t test, $p < 0.001$) for both the MITH ($m = 0.58$, $r^2 = 0.972$) and FAT ($m = 0.66$, $r^2 = 0.946$) methods, but no significant difference was detected between the slopes or elevation of the lines describing these relationships (Student's t test, $p < 0.01$). When pHi values obtained from each method were regressed against each other (Fig. 2.3), a statistically significant linear relationship (Student's t test, $p < 0.001$) was observed that was not significantly different from a slope of 1 ($m = 0.82$, $r^2 = 0.95$, $p < 0.01$).

2.5 Discussion

This study used RBCs as a representative tissue to test the effects of storage duration and preparation associated with the MITH method on pHi values of hypercarbic tissues. As separated RBCs have no extracellular component, determining the effect of diffusive CO_2 loss on pHi was, perhaps, less complex than in other tissues. However, the factors that are important for assessing the effect of diffusive CO_2 loss on pHi (i.e., CO_2 gradients, storage time, and surface area to volume ratio) were not altered in the use of RBC as a proxy for other tissues. Consequently, this study clearly demonstrates that storage of tissues obtained from fish exposed to 30 mm Hg PCO_2) and maintained in LN_2 for up to 30 days has no effect on pHi; however, a small increase in pHi was observed after 90 days of storage (Fig. 2.1), regardless of the storage method (i.e., LN_2 or ultracold (-80°C) freezer). While this difference was statistically significant, a 0.025 pH increase represents a small proportion (< 5%) of the acidosis associated with the treatment (0.6

pH units). Thus, within 90 days, the particular method of freezing and storage duration appears to have little relative effect on the obtained pHi value.

More importantly, pHi measured using the MITH method of Pörtner et al. (1990) returned values similar to those obtained by the previously validated FAT method in red blood cells exposed to hypercarbia up to 75 mm Hg PCO_2. Consequently, the MITH method is, based on these experimental data, suitably accurate for determination of pHi from excised tissues in fishes exposed to aquatic hypercarbia up to and including 75 mm Hg PCO_2.

2.6 Figures

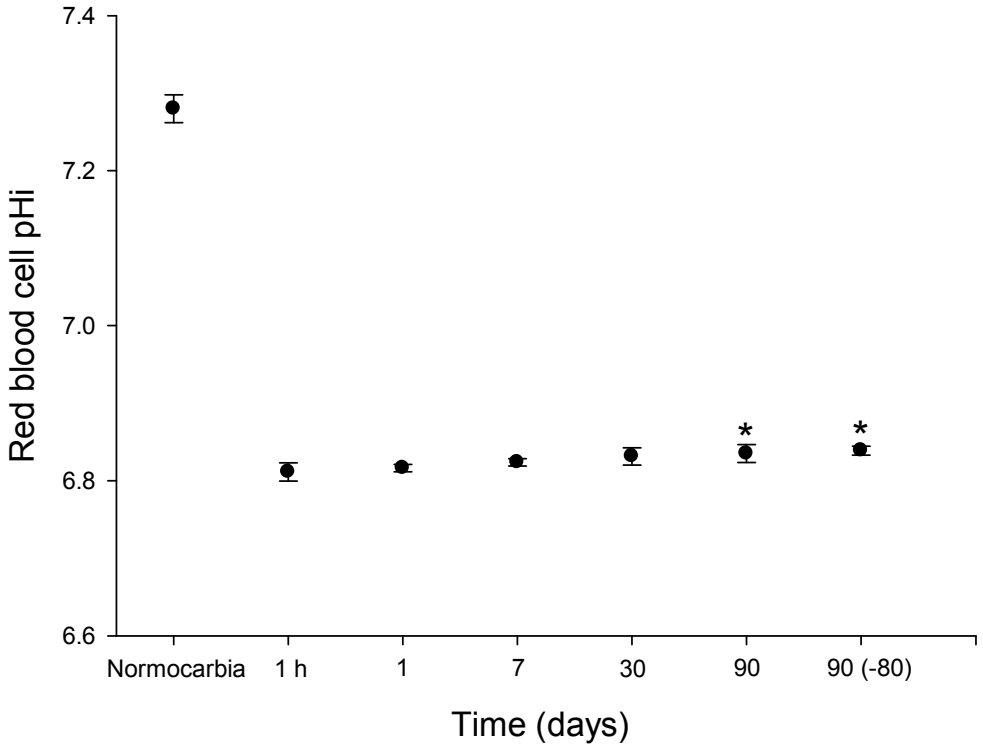


Figure 2.1 The effect of duration (days) of storage in liquid nitrogen (LN_2) on red blood cell (RBC) pHi in white sturgeon exposed to 30 mm Hg PCO_2 for 48 h. The group furthest to the right represents the mean pHi value of RBC frozen in LN_2 and transferred immediately to an ultracold (-80°C) freezer for 90 days. Values are means ± s.e.m. An asterisk indicates a significant difference from groups measured prior to storage. RBC pHi was measured *via* the freeze-thaw method (FAT) of Zeidler and Kim (1977).

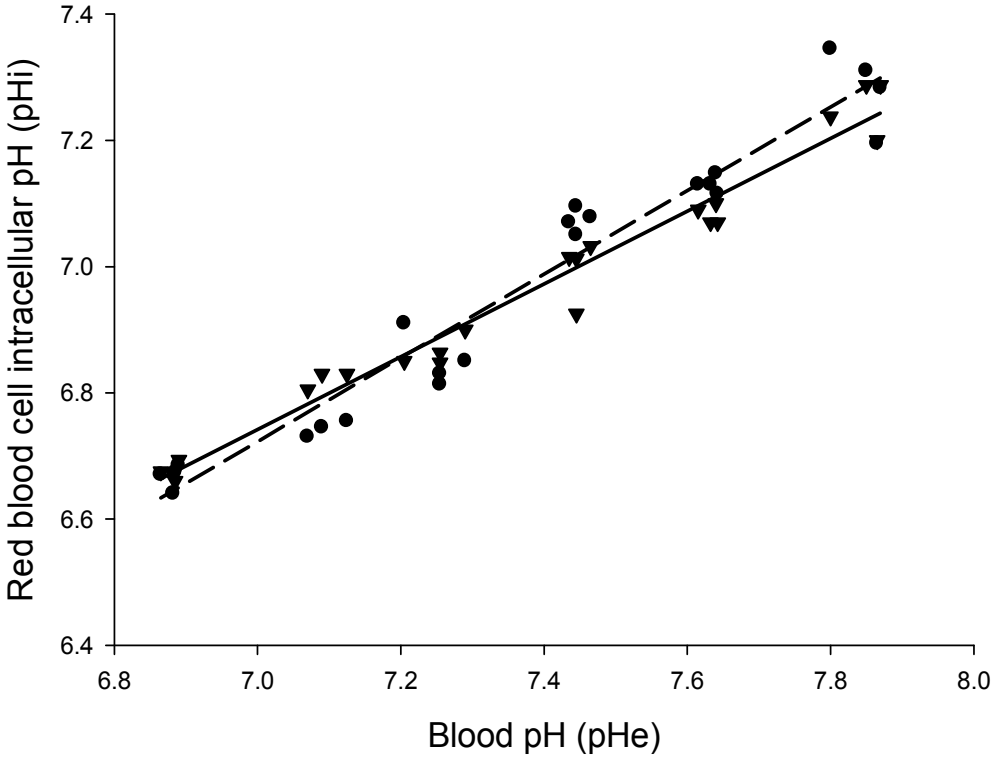


Figure 2.2 The relationship between pHe and pHi in response to different levels of CO_2 as described by either the freeze-thaw method (FAT) (circles) or the metabolic inhibitor tissue homogenate (MITH) method (triangles). Raw data are shown. The slopes and the elevations of the regression lines for FAT (dashed line; m = 0.66, r^2 = 0.946) and MITH (solid line; m = 0.58, r^2 = 0.972) are not significantly different from each other.

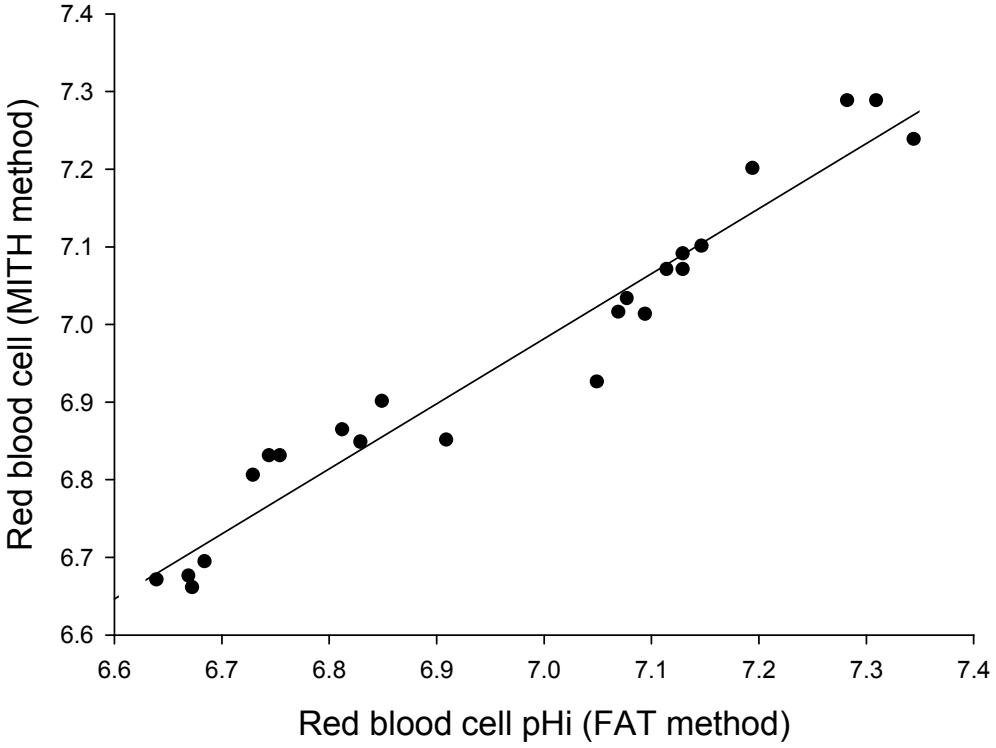


Figure 2.3 The correlation between pHi measured *via* the freeze-thaw method (FAT) or the metabolic inhibitor tissue homogenate (MITH) method. The slope of the line for this correlation is not significantly different from 1 ($m = 0.84$, $r^2 = 0.945$, $p < 0.001$).

3: COMPLETE INTRACELLULAR pH PROTECTION DURING EXTRACELLULAR pH DEPRESSION IS ASSOCIATED WITH HYPERCARBIA TOLERANCE

3.1 Synopsis

Sturgeons are among the most CO_2-tolerant of fishes investigated to date. However, the basis of this exceptional CO_2 tolerance is unknown. Here, white sturgeon, *Acipenser transmontanus*, were exposed to elevated CO_2 to investigate the mechanisms associated with short-term hypercarbia tolerance. During exposure to 11.5 mm Hg PCO_2, pHe depression was compensated within 24 h and associated with net plasma HCO_3^- accumulation and equimolar Cl^- loss. Moderate hypercarbia also induced 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 branchial mechanisms involved may differ. White sturgeon exposed to 48 h of severe hypercarbia (22.5 and 45 mm Hg PCO_2) 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 11.5 mm Hg PCO_2) or prolonged (48 h at 22.5 and 45 mm Hg PCO_2) blood acidosis. This pHi protection was greater than that estimated to be attributable to intrinsic buffering in tissues. Such tight active cellular regulation of pHi in the absence of pHe compensation represents a novel pattern for water-breathing fishes, and I hypothesize that it is the basis for the exceptional CO_2 tolerance of white sturgeon and, perhaps, other CO_2-tolerant fishes.

3.2 Introduction

Aquatic hypercarbia (elevated PCO_2 in water) occurs in freshwater and estuarine systems, and water PCO_2 levels as great as 60 mm Hg (20-30 fold increase over the resting arterial PCO_2 of fish) have been observed (Heisler, 1982; Ultsch, 1996) in tropical freshwater environments. Exposure to elevated aquatic PCO_2 induces physiological and morphological changes that have been reasonably well described in a few species of teleost (Chapter 1). In brief, the initial rapid respiratory acidosis is corrected by a more gradual metabolic alkalosis, which may, over days, return pHe and pHi to pre-exposure [i.e., normocarbic levels]. This pHe recovery is achieved primarily at the gill [80-95%, Perry et al., 1987; Perry and Gilmour, 2006] and is accompanied by morphological [e.g., reduced apical surface area of mitochondrial rich cells (MRC) and increased surface area of pavement cells (PVC) (Goss et al., 1998)] and molecular [changes in activity or expression of, for example, the proton extruding pump V-type H^+-ATPase (V-ATPase) (Lin et al., 1994), or Na^+/H^+-exchangers (NHE) (Edwards et al., 2005)] changes at the gill. As some MRC are hypothesized to be the site of base uptake, and PVC to excrete acid, these changes in branchial morphology and acid-base relevant ion transporters may aid with the net acid efflux necessary to promote blood pH compensation (Edwards et al., 2005; Goss et al., 1998), 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 (Chapter 1; Putnam and Roos, 1997; Brauner and Baker, 2009). 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 (Heisler, 1999; Putnam and Roos, 1997). 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 (reviewed in Chapter 1; Brauner and Baker, 2009), although a few instances of more rapid tissue pHi recovery exist [e.g., gill in rainbow trout, (Wood and LeMoigne, 1991); brain and heart in little skate (Wood et al., 1990)].

As hypercarbia and the resulting acidosis increase in severity, complete pHe recovery becomes limited due to the "bicarbonate concentration threshold" (Chapter 1; Heisler, 1999; Brauner and Baker, 2009). During short term exposure (days) to hypercarbia, most fish species studied to date are not capable of increasing plasma HCO_3^- (in exchange with Cl^-) greater than 27 - 33 mmol l^{-1}, and thus cannot fully compensate for the respiratory acidosis associated with CO_2 levels greater than approximately 15 mm Hg PCO_2 (Chapter 1; Heisler, 1999; Brauner and Baker, 2009); this failure is associated with morbidity, although surprisingly little research has described CO_2 related toxicity (but see Chapter 4; Hayashi et al., 2004; Ishimatsu et al., 2004).

Despite limits to pHe compensation, a few fish species (e.g., European eel, *Anguilla anguilla*, McKenzie et al., 2003) are able to tolerate exposures much greater than 15 mm Hg PCO_2, in the face of prolonged pHe depression. Two of these tolerant species, the facultative air breathers, *Synbranchus marmoratus* (Heisler, 1982) and *Pterygoplichthys pardalis* (Brauner et al., 2004) have demonstrated an ability to protect pHi in some tissues (e.g., heart and white muscle) during a prolonged and severe blood acidosis. White sturgeon (*Acipenser transmontanus* Richardson 1836) can tolerate levels of hypercarbia that induce a severe blood acidosis for days (Crocker and Cech, 1998). I hypothesize that exceptional hypercarbia tolerance of white sturgeon is associated with a capacity for preferential pHi regulation during hypercarbia (i.e., the ability to tightly regulate pHi in tissues during a pHe depression).

The objective of this study was to investigate acid-base regulation in white sturgeon during hypercarbic challenges both within and beyond the pHe compensatory capacity of most fish. To this end, I exposed white sturgeon to water equilibrated with 1.5, 3 and 6% CO_2 by volume (11.5, 22.5, and 45 mm Hg PCO_2). To elucidate the mechanisms of pHe compensation

during hypercarbia, I measured changes in blood physiology (e.g., pHe, $[HCO_3^-]$ 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 11.5 mm Hg PCO_2. To test my hypothesis that white sturgeon protect tissue pHi during an extracellular acidosis, I characterized pH changes in tissues (including RBC, heart, liver, brain, and white muscle) in response to the hypercarbic challenges described above. Finally, I calculated intracellular intrinsic (i.e. non-bicarbonate) buffer values from homogenized white sturgeon tissues (i.e., white muscle, heart, and liver) to estimate the importance of active pH regulatory mechanisms involved in pHi regulation.

3.3 Materials and methods

3.3.1 Animals and rearing conditions

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) in Nanaimo, B.C., Canada. Experiments in Series 1 (see below) were performed at VIU in the fall with 3 year old white sturgeon (length ~ 50-80 cm, mass ~ 1-2.5 kg), where water was very soft and dilute (hardness: 12 μmol l^{-1} $[CaCO_3]$, alkalinity: 13-14 μmol l^{-1}, pH: 6.6-6.9, $[Na^+]$ and $[Cl^-]$ less than 1 mg l^{-1} each). For Series 2, white sturgeon (4 years old, mass ~ 1-2 kg) initially spawned at VIU were obtained from Target Marine Hatchery (Sechelt, B.C., Canada), and held at the University of British Columbia (UBC), Vancouver, B.C., for several months prior to experimentation. Water at this facility was even softer (water hardness: 4 μmol l^{-1} $[CaCO_3]$, alkalinity: 3-4 μmol l^{-1} $[CaCO_3]$, pH: 6.7-7.0, $[Na^+]$ and $[Cl^-]$ less than 3 mg l^{-1} each).

All animals were held in large, outdoor flow-through tanks (PwO_2 > 130 mm Hg, $PwCO_2$ < 0.1 mm Hg, T=11-13ºC, 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 CO_2 levels used in this study in the 3 month 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 animals were held and used according to regulations laid out by the Canadian Animal Care Committee (UBC AC 02-0222, MUC 2004-04R).

3.3.2 Series 1: The effect of hypercarbia (11.5 mm Hg PCO_2) on blood and tissues

White sturgeon were placed into a re-circulating system (PwO_2 > 135 mm Hg, and $PwCO_2$ < 0.1 mm Hg PCO_2, consisting of darkened plastic boxes (30 l, flow rate ~ 3 l min^{-1}, 17ºC), for 24 h prior to experiments. This period is sufficient to allow recovery from handling stress in sturgeon (e.g., Crocker et al., 2000; Baker et al., 2005a; Baker et al., 2005b). Normocarbic white sturgeon were terminally sampled immediately following this acclimatization period (control or pre-exposure group). Sturgeon were then exposed to either a further 48 h of normocarbia, or to $PwCO_2$ ~ 11.5 mm Hg PCO_2 for 6, 24, or 48 h, which was induced by plumbing a mixing tank into the re-circulating system and bubbling it with pre-set rates of air and 100% CO_2 using Sierra Instruments mass flow controllers. $PwCO_2$ was measured with a PCO_2 electrode to confirm target CO_2 tensions. Water oxygen levels remained high throughout all treatments (PwO_2 > 115 mm Hg).

Following exposure, each box was isolated from the recirculation system, and the animals were euthanized with MS-222 (0.3 g l^{-1}, buffered with $NaHCO_3$). 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 (150 i.u. ml^{-1} heparin) rinsed syringe (10 ml syringe,

23G1 needle), and placed on ice. Following this procedure (< 1 min), fish were killed *via* spinalectomy, and the following tissues were excised, placed in pre-labelled aluminum foil, 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 2nd and 3rd gill arches (left side). All freeze-clamped tissues were then stored at -80°C. Next, 2nd and 3rd gill arches (right side) were removed and stored in either Karnovsky's solution for electron microscopy (2nd arch), or 3% paraformaldehyde in phosphate buffered saline (PBS) for immunofluorescence microscopy (3rd arch). Blood was divided into two equal aliquots. Haemoglobin concentration ([Hb]), haematocrit (Hct) and mean cell haemoglobin concentration (MCHC) were measured from one aliquot, and from the other, blood pH (pHe), plasma total carbon dioxide (TCO_2), and plasma ions (Na^+, Cl^-, Mg^{2+}, and Ca^{2+}) were measured, as described below.

3.3.3 Series 2: The effect of hypercarbia (22.5 and 45 mm Hg PCO_2) on blood and tissues

White sturgeon were anaesthetized (MS-222, 0.2 g l^{-1}, buffered with $NaHCO_3$), transferred to a surgical table, and, while gills were irrigated with an oxygenated MS-222 (0.05 g l^{-1} buffered with $NaHCO^3$) solution, a dorsal aortic catheter (PE 50, Intramedic) was surgically implanted as has been previously described (Crocker and Cech, 1998). Following surgery, each cannulated sturgeon was transferred to a black box (30 l) supplied with re-circulated aerated water (flow rate > 3 l min^{-1}, T = 13ºC). Each cannula was flushed daily and following sampling with lithium-heparinized (20 i.u. ml^{-1} heparin) 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 [HCO_3^-], [Hb], Hct, MCHC, and [Cl^-] were measured as described below. RBC pHi was measured where volume obtained permitted. Fish were then exposed to water equilibrated with one of

three CO_2 tensions: 1) normocarbic water (air saturated), 2) 22.5 mm Hg PCO_2 or 3) 45 mm Hg PCO_2. Water PCO_2 was verified *via* a thermostated (13ºC) Radiometer PCO_2 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 hours, 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 pHi and non-bicarbonate buffering capacity.

3.3.4 Analytical techniques

Haemoglobin concentration (using Drabkin's reagent), haematocrit and RBC MCHC were determined as described previously (Baker et al., 2005a; Baker et al., 2005b). Blood glucose was measured with a blood glucose meter (Ascensia Elite, Bayer). Blood pH was measured using a thermostated capillary pH electrode (Radiometer, BMS 3 MK 2). The remaining blood was centrifuged (3 min@10,000 rpm), and plasma was removed for measurement of total CO_2 content (TCO_2 Analyzer, Corning, Model 965), osmolarity (Westcor Vapor Pressure Osmometer, Model 5520), and inorganic ions ($[Na^+]$, Flame photometer, Corning, Model 410; $[Cl^-]$ HBI Digital Chloridometer, Model 4425000; $[Ca^{2+}]$ and $[Mg^{2+}]$, flame spectrophotometer, Varian, AA 240 FS). Blood PCO_2 and plasma $[HCO_3^-]$ were calculated from total CO_2 and pH measurements as described previously (Brauner et al., 2004), using the CO_2 solubility coefficient (αCO_2) and pK' for rainbow trout (Boutilier et al., 1984) and a re-organization of the Henderson-Hasselbach equation. This indirect method has been used previously for fish exposed to hypercarbia (Brauner et al., 2004), but assumes PCO_2 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 pHi using the freeze thaw technique (Milligan and Wood, 1985; Zeidler and Kim, 1977). Tissues were later ground under liquid nitrogen and intracellular pH was measured using the metabolic inhibitor tissue homogenate method (Pörtner et al., 1990), which I have verified to be accurate in tissues exposed to higher PCO_2 tensions (Baker et al., 2009b; Chapter 2). Tissue $[HCO_3^-]$ was calculated as in blood, using pK' values from previous research (Boutilier et al., 1984), and assuming PCO_2 to be in equilibrium between water, blood, and tissues, as has been assumed in previous work (Heisler, 1982; Boutilier et al., 1984; McKenzie et al., 2003). RBC and tissue pHi was measured using the same thermostated electrode as that described above for blood.

Non-bicarbonate 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 3.75, 7.5, 15, 30, 45, or 75 mm Hg PCO_2 using a Wösthoff (DIGAMIX 6KM 422) gas mixing pump (n = 4 for each CO_2 level). At each CO_2 level, plasma TCO_2 and pH were measured as described above. Tissue non-bicarbonate buffer capacity was assessed similarly, but using a modified version of the previously described CO_2-equilibrated tissue homogenate technique (Hansen and Gesser, 1980; Heisler, 1982). In brief, freeze-clamped white sturgeon heart, liver or white muscle was pulverized in a liquid nitrogen cooled mortar and pestle, suspended in an iso-osmotic (0.9%) KCl solution, vortexed gently until homogeneous and centrifuged at low speed to remove cellular debris. The supernatant was equilibrated at 2, 7.5, 15 and 30 mm Hg PCO_2 and sampled for pH and TCO_2 as described above for blood. Intracellular fluid volume was determined using the difference between total tissue water fraction (measured from wet and dry weight of respective tissues), and previously determined extracellular fluid volume (Munger et al., 1991). CO_2 solubility constants and pK' were calculated using equations from previous work (Brauner et al., 2004). In both cases, non-bicarbonate buffer capacity (β_{NB}) was calculated from the slope of $\Delta[HCO_3^-]$ ΔpH^{-1}, with intracellular β_{NB} also corrected for

buffering due to both extracellular fluid and dilution medium, and then expressed in mmol HCO_3^- pH^{-1} l^{-1} of blood or kg^{-1} of intracellular tissue water, over an *in vivo* relevant pH range.

3.3.5 Scanning electron microscopy

After fixation in Karnovsky's solution, gill arches (n = 4 for each treatment) were post-fixed in 1% osmium tetroxide, completely dehydrated in ethanol, and critical-point-dried with liquid CO_2. Gill arches were mounted with the lateral side of the filament parallel to surface, and sputter-coated with gold. Afferent or trailing edges (TE) of the filament were examined with a scanning electron microscope (Hitachi S 2700, Tokyo, Japan). Mitochondria-rich cell density (i.e., number of MRC per mm^2) on the outermost layer of the filament epithelium was counted on randomly selected photographs at a magnification of 2000x in 3 non-contiguous areas of 5 different filaments per fish and 3 fish per group. The apical surface area of individual MRCs was calculated on microphotographs at 5,000X magnification according to the shape of their two-dimensional apical openings that varied from circular to trapezoidal. The fractional surface area of MRC (FAMRC) was estimated by the previously described weighting method (Talikina et al., 2001). The density of microridges on pavement cell (PVC) surface was calculated on the microphotographs at 5,000X magnification by counting intercepts of microridge profiles with segments of a test grid superimposed on the photographs (Goss et al., 1998). Microphotographs were taken of the PVC surface with the highest density of microridges.

3.3.6 ATPase assay

Gill Na^+,K^+-ATPase (NKA) and V-ATPase activities were measured using a kinetic microassay at 25°C (McCormick, 1993) as modified by Wilson et al. (2007). Total protein was

measured using the Bradford dye binding assay (Bio-Rad, Hercules, CA, USA) with a bovine serum albumin (BSA) standard. Ouabain (1.0 mmol l^{-1}; Sigma–Aldrich Chemical Co., St. Louis, MO, USA) and bafilomycin A1 (10 μmol l^{-1}; LC Laboratories, Woburn, MA, USA) were used in the ATPase assays to specifically inhibit NKA and V-ATPase activities, respectively. Activities are expressed in μmol ADP mg^{-1} protein h^{-1}.

3.3.7 Immunoblotting

Immunoblotting was carried out as described previously (Wilson et al., 2007). Briefly, samples were electrophoretically separated by SDS-PAGE (10% T), and transferred to PVDF membranes by semi-dry electrophoretic transfer. Membranes were then blocked with 5% blotto in TTBS for 1 h, and probed overnight with primary antibody diluted 1:1000 [mouse monoclonal anti-Na^+/K^+-ATPase α subunit: clone α5; rabbit polyclonal anti-V-ATPase B subunit: B1/B2 VATP (Wilson et al., 2007) or rat polyclonal anti-NHE3: R1B2 (Choe et al., 2005) antibodies]. Following washes in TTBS, membranes were incubated with goat anti-rat, mouse or rabbit HRP conjugated secondary antibody (100 ppm) and labelling was detected by enhanced chemiluminescence (ECL; Immobilon, Millipore) using a CCD imaging system (LAS 4000 mini Fujifilm, Tokyo Japan). Bands were quantified using Fujifilm Science Lab software.

3.3.8 Immunofluorescence microscopy

Gills fixed in 3% PFA/PBS for 24 h were paraffin embedded and sectioned for indirect immunofluorescent localization of NKA, V-ATPase and NHE3 as described previously (Choe et al., 2005; Wilson et al., 2007). The sections were pre-treated with 0.05% citraconic anhydride (pH 7.4) for 30 min at 95°C (Namimatsu et al., 2005), and then rinsed and probed with mouse

monoclonal NKA α subunit antibody (α5) and either rabbit anti-peptide V-ATPase B subunit polyclonal antibody (B1/B2 VATP) or rat polyclonal anti-NHE3 antibody (R1B2) which had been diluted 1:200 in 1% BSA/TPBS overnight. The slides were rinsed in TPBS and then incubated with goat anti-mouse Alexa Fluor 594 and goat anti-rabbit or anti-rat Alexa Fluor 488 conjugated secondary antibodies (Molecular Probes Inc, Eugene, OR, USA), both diluted 1:200 in BSA/TPBS for 1 h at 37°C. The nuclei were stained with DAPI (Molecular Probes), rinsed with TPBS, and cover slips were mounted with 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 mol l^{-1} Tris (pH 8.5). The sections were viewed on a Leica DM6000B wide field epi-fluorescence microscope and images captured using a digital camera (Leica DFC 340 FX, Lisbon, Portugal). Preliminary testing with single antibodies was conducted as well as with negative controls (peptide blocking, normal host sera and culture supernatant).

3.3.8 Statistical analyses

All values are presented as mean values ± s.e.m, and sample sizes are indicated in parentheses in text (e.g., n = 6). Changes in water, blood and tissue parameters in Series 1 were analyzed using one-way analysis of variance (one-way ANOVA). In Series 2, the effects of exposure time and CO_2 level on blood parameters were analyzed using two-way repeated measures (RM) ANOVA. Where a significant interaction was observed, either one-way RM ANOVA (effect of time within treatments) or one-way ANOVA (effect of CO_2 treatment at each time) was used to determine effects. In all cases, an alpha of 5% ($p < 0.05$) was selected to signify statistically significant differences. Where differences were indicated, Tukey's or Dunnett's post-hoc tests (as appropriate) were used to determine homogeneous subsets. Where normality or equal variance assumptions were violated, non-parametric ANOVA on ranks was performed. Relationships between blood pH and intracellular pH in tissues of individuals were

described using correlation analysis ($\alpha = 0.05$). All statistical tests were performed with SPSS (ver. 11.0).

3.4 Results

3.4.1 Series 1: The effect of hypercarbia (11.5 mm Hg PCO_2) on blood and tissues

White sturgeon exposed to 11.5 mm Hg PCO_2 exhibited an increase in blood PCO_2 (2.9 ± 0.5 to 11.6 ± 0.4 Torr, $p < 0.05$, Fig. 3.1A). Blood pH decreased significantly in these fish after 6 h, but after 24 h, it was no longer significantly different from control fish (Fig. 3.1A). Mean plasma [HCO_3^-] was significantly higher (Fig. 3.1A), and mean plasma [Cl^-] was significantly lower following 6, 24 and 48 h of this CO_2 exposure than in controls (Table 3.1). Plasma [Na^+], [Mg^{2+}], and osmolality did not change significantly, although plasma [Ca^{2+}] was significantly higher following hypercarbia exposure (Table 3.1). [Hb] (pooled value: 0.85 ± 0.03 mmol l^{-1}), HCT (pooled value: 33.2 ± 0.9%), and MCHC (pooled value: 2.53 ± 0.02 mmol kg^{-1} packed RBCs) of hypercarbic sturgeon were not significantly different from either pre-exposed sturgeon or sturgeon exposed to 48 h of normocarbia. Blood glucose of hypercarbic fish was significantly higher at 6 h compared to 24 and 48 h (Table 3.1). RBC pHi was significantly depressed following 6 h, but not 24 (Fig. 3.1B); at 48 h, pHi was significantly elevated. White muscle pHi did not change significantly (pooled value: 7.20 ± 0.02), although white muscle [HCO_3^-] was elevated at 24 and 48 h (Table 3.2). Brain, liver and heart pHi (Fig. 3.1C) and [HCO_3^-] (Table 3.2) were significantly elevated over respective control tissue values following 6, 24 and 48 h of exposure. Blood pH (pHe) was significantly correlated with RBC pHi (slope = 0.48, $r^2 = 0.26$, $p < 0.05$), but not with white muscle, brain, heart or liver pHi.

In control fish, pavement cells (PVC) comprised about 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. 3.2B, 3.3A). The apical surface of the MRC varied in appearance, size and topography such that there appeared to be two distinct populations. Apices of about 67% of MRC were large, slightly convex and ornamented with either long, thin and ramified microvilli, or shorter and thicker microvilli (referred to as MRCLA, Figs. 3.2B-D, 3.3A). The rest of the MRC (MRCSA) exhibited small, mostly circular, flat or slightly convex apical surface, with short microvilli (Fig. 3.3A).

Exposure to 11.5 mm Hg PCO_2 caused morphological modifications in both PVC and MRC. In PVCs, the most complex "lace-like" patterns and, correspondingly, the highest density of branched and interdigited microridges were observed after 6 h of hypercarbia (Figs. 3.3B, 3.4A). After 48 h, microridge density was no longer significantly higher than control (Figs. 3.3D, 3.4A). Cellular borders between PVC became clearly defined with tall parallel microridges (Fig. 3.3D). Density and total surface area of MRCLAs was significantly reduced during this CO_2 challenge (Figs. 3.3B-D, 3.4B-D). After 6 h, density of MRC decreased almost 30% below control (Fig. 3.4B), and the fractional area of MRC (FAMRC) decreased to less than half that of control values (Fig. 3.4C). Surface area of individual MRC at 6 h was significantly lower than in normocarbic gills, and decreased further by 24 h of exposure to 11.5 mm Hg PCO2 (Fig. 3.4C).

NKA activity increased significantly after 48 h exposure to 11.5 mm Hg PCO_2 (Fig. 3.5A); however, western blots indicated NKA α subunit protein levels did not change (Fig. 3.5C). A single band of approximately 100 kDa was identified in gill homogenates. V-ATPase activity and B subunit protein levels were unaffected (Fig. 3.5B, D). A single band of approximately 56 kDa was recognized by the B1/B2 VATP polyclonal antibody. The NHE3

R1B2 antibody weakly cross-reacted with bands at ~75 and ~50 kDa (Fig. 3.5E), but no changes in either band were observed during exposure to 11.5 mm Hg PCO_2.

In gills from normocarbic sturgeon, the NKA α subunit was immunolocalized to cells in the interlamellar and lamellar epithelia (Fig. 3.6A, D). Labelling 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 NKA immunoreactive cells (Fig. 3.6B,C). There was also some weaker cytoplasmic staining in these cells. In general, the NHE3 immunoreactivity (IR) was weak and required longer exposure times; thus, background staining was more of a problem. The labelling of erythrocytes, which was nonspecific as determined by control staining, was particularly noticeable. V-ATPase immunofluorescence was generally found in gill cells without detectable NKA IR; in some cells, however, there was co-localization of staining (Fig. 3.6D-F). V-ATPase IR 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 NKA, NHE3 and V-ATPase did not change appreciably with 48h of exposure to 11.5 mm Hg CO2.

3.4.2 Series 2: The effect of hypercarbia (22.5 and 45 mm Hg PCO_2) on blood and tissues

Normocarbia-exposed sturgeon exhibited no significant changes in pHe, plasma [HCO_3^-] or plasma [Cl^-] over the course of the 48 h experiment. Within 15 min of exposure to 22.5 and 45 mm Hg PCO_2, pHe decreased and remained significantly different throughout the 48 h exposure. Partial pHe recovery did occur within each treatment by comparison to the lowest pH values measured at 3 h (Fig. 3.7A). Plasma [HCO_3^-] was significantly elevated at 1 h compared to time 0, and remained so throughout exposure to both 22.5 and 45 mm Hg PCO_2 (Fig. 3.7B). Plasma

[Cl^-] decreased significantly after 24 h of exposure to 22.5 mm Hg PCO_2 (141 ± 4 to 131 ± 4 mmol l^{-1}) and 48 h of exposure to 45 mm Hg PCO_2 (152 ± 6 to 123 ± 5 mmol l^{-1}). White sturgeon HCT (pooled value 28.8 ± 0.4%), [Hb] (pooled value 0.75 ± 0.02 mmol l^{-1}), and MCHC (pooled value 2.63 ± 0.04 mmol kg^{-1} 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 pHi was significantly depressed in fish exposed to hypercarbia at all time points compared to RBC pHi of normocarbia exposed fish (Fig. 3.8A), despite some pHi recovery after 48 h. When pHe was regressed against RBC pHi over the course of the experiment (Fig. 3.8B), or at 48 h (Fig. 3.9A), a statistically significant positive correlation was found. However, mean pHi of heart, liver, brain and white muscle of white sturgeon was not significantly different between treatments, despite the substantial difference in pHe between treatments (Fig. 3.8). Furthermore, pHe regressed against pHi in heart, brain, liver and white muscle after 48 hours of hypercarbia did not exhibit the positive relationship observed for RBC pHi (Fig. 3.9A, B). Tissue [HCO_3^-] was elevated following 48 h of both CO_2 treatments (Table 3.2).

Increasing CO_2 levels altered both blood (Fig. 3.10), and tissue (Fig. 3.11A-C) pH and [HCO_3^-]. Blood non-bicarbonate buffer capacity was -11.9 mmol HCO_3^- pH $unit^{-1}$ l^{-1} blood (r^2 = 0.88). Non-bicarbonate (i.e., intrinsic) buffer capacity in white muscle, heart, and liver of white sturgeon (as calculated from Figure 3.11 A-C over an *in vivo* relevant pH and CO_2 range) was -35.3, -11.3, and -8.9 mmol HCO_3^- pH $unit^{-1}$ kg^{-1} intracellular tissue water, respectively.

3.5 Discussion

White sturgeon responded to a respiratory acidosis induced by exposure to 11.5 mm Hg PCO_2 with pHe compensation qualitatively similar to most other fishes examined (e.g., Lloyd and White, 1967; Cameron and Randall, 1972; Perry et al., 1987a). Changes in gross gill morphology were also similar to those previously observed in teleosts (Goss et al., 1998). However, patterns of activity and expression of branchial ionoregulatory transporters during hypercarbia were different in white sturgeon compared to other teleosts studied (Evans et al., 2005), as V-ATPase activity did not increase. When exposed to 22.5 and 45 mm Hg PCO_2, pHe recovery was incomplete. Nevertheless, pHi was preferentially regulated at normocapnic pH levels in the heart, liver, brain and white muscle (but not RBC) during hypercarbia. This pHi homeostasis exceeded intrinsic (i.e., non-bicarbonate) intracellular buffering, and therefore is most likely due to active cellular trans-membrane acid-base ion transport. This active pHi regulatory capacity, particularly in the brain, liver and heart, likely represents the basis for the exceptional tolerance of sturgeon to short-term severe CO_2 exposure.

3.5.1 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., Baker et al., 2005a; Baker et al., 2005b). Most values from normocarbic white sturgeon tissues in this study were consistent with those observed in teleosts [e.g., cod, *Gadus morhua* (Larsen et al., 1997), *P. pardalis*, (Brauner et al., 2004), *S. marmoratus* (Heisler, 1982), and rainbow trout (Wood and LeMoigne, 1991)], although there were a few discrepancies [e.g., rainbow trout (Wood and LeMoigne, 1991) and sea raven (*Hemitripterus americanus*, Milligan and Farrell,

1986) hearts]. Brain pHi has rarely been measured in fishes, but values for normocarbic trout (Wood and LeMoigne, 1991) are higher (0.2-0.3 pH units) than values for the white sturgeon in this study.

3.5.2 pHe recovery during moderate hypercarbia

This study assessed the capacity of white sturgeon to alter net epithelial acid-base relevant ion transport to drive pHe recovery during hypercarbia (11.5 mm Hg PCO_2). Changes in white sturgeon acid-base physiology during a moderate hypercarbic challenge (11.5 mm Hg PCO_2 over 48 h) were qualitatively similar to those reported in most teleosts (e.g., Wood and LeMoigne, 1991; Goss et al., 1998) and elasmobranchs (e.g., Heisler, 1988, Graham et al., 1990) investigated to date. White sturgeon exhibited rapid recovery associated with a net elevation in plasma [HCO_3^-] matched by an equimolar reduction in plasma [Cl^-]. The time course of pHe recovery was similar, if perhaps more rapid than that observed in rainbow trout exposed to a similar CO_2 challenge (Larsen and Jensen, 1997).

Blood pH compensation at 11.5 mm Hg 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 interdigited microridges (Figs. 3.3, 3.4A). 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, Goss et al., 1998). Concurrently, the fractional surface area of MRC (FAMRC) exhibited a progressive reduction: after 48 h, this decrease was almost 50% (Figs. 3.3, 3.4D). This was due to all of the following: i) a decrease in the number of apically exposed MRC (Fig. 3.4B), ii) morphological alteration of mitochondrial rich cells with a larger surface area (MRCLA) to mitochondrial rich cells with a smaller surface area (MRCSA), and iii)

reduction of surface area of MRC (Fig. 3.4C). As MRC may be the most important site of chloride uptake in fresh water fishes (Goss et al., 1998; Evans et al., 2005), these changes which have also been observed in teleosts exposed to hypercarbia (Goss et al., 1998), are hypothesized to reduce sites of chloride uptake or base excretion (Evans et al., 2005; Goss et al., 1998). NKA, heavily concentrated on the basolateral membrane of MRC, exhibited increased activity during hypercarbia (Fig. 3.5A), and, as NKA was found co-localized with V-ATPase in some branchial sites (Fig. 3.6D-F), this finding could imply a contribution of this enzyme to branchial or whole animal pH homeostasis.

In contrast, exposure to 11.5 mm Hg PCO_2 did not induce changes in NHE3 protein levels or either V-ATPase activity or V-ATPase B subunit expression in white sturgeon (Fig. 3.5D). In rainbow trout, *O. mykiss*, increases in V-ATPase activity and expression have been observed in response to hypercarbia [NEM-sensitive proton ATPase activity (Lin and Randall, 1993); V-ATPase E subunit expression in immunoreactive (IR) cells (Sullivan et al., 1995); V-ATPase A subunit expression in IR cells, (Lin et al., 1994)]. 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 (Lin et al., 1994; Sullivan et al., 1995). It is also possible that existing transporters are sufficient to drive pHe compensation, as increases in NHE3 expression are not always seen in response to hypercarbia [e.g., Atlantic stingray, *Dasyatis sabina*, (Choe and Evans, 2003); freshwater acclimated killifish, *F. heteroclitus*, (Edwards et al., 2005)]. A third possibility is that NHE3 and V-ATPase play a limited role in branchial pH compensation of white sturgeon to hypercarbia. If this were the case, white sturgeon could be considered a candidate for investigation into branchial HCO_3^-/Cl^- exchangers, such as those that have been hypothesized to be involved in

pHe compensation during hypercarbia in, for example, the Atlantic stingray (Piermarini et al., 2002; Choe and Evans, 2003), as no Cl^-/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 (Evans et al., 2005; Perry and Gilmour, 2006). 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 less than 10% of net acid production *via* urine (Perry and Gilmour, 2006), this has not been confirmed in sturgeon.

Despite the relatively rapid pHe compensation in white sturgeon during exposure to 11.5 mm Hg PCO_2, in white sturgeon exposed to severe hypercarbia (22.5 and 45 mm Hg PCO_2), blood pH remained depressed for 48 h (Fig. 3.7A,B). In a previous study (Crocker and Cech, 1998), white sturgeon exposed to 30 mm Hg PCO_2 also exhibited a blood acidosis and little pHe compensation (pH recovery of approximately 20% after 96 h). This is consistent with an apparent limitation to pH compensation observed in other fishes (Chapter 1; Heisler, 1999; Brauner and Baker, 2009,). However, during these exposures white sturgeon bicarbonate accumulation did not reach the proposed "bicarbonate concentration threshold" (i.e., 27-33 mmol l^{-1}), as net plasma [HCO_3^-] did not exceed 20 mmol l^{-1} at 22.5 or 45 mm Hg PCO_2. The limit for net HCO_3^- accumulation 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 approximately 5.5 and 4.5 at 22.5 and 45 mm Hg 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 (Lin and Randall, 1993). 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).

3.5.3 pHi during hypercarbia exposure

In this study, white sturgeon RBC pHi exhibited a qualitatively similar pattern of change to whole blood pH during hypercarbia exposure; as pHe recovered, so did RBC pHi (Figs. 3.1B, 3.8A,B, 3.9). When RBC pHi values for all CO_2 tensions and times were plotted against blood pH, 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 β-NHE (Brauner and Berenbrink, 2007). 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 root effect haemoglobins (Brauner and Berenbrink, 2007; Regan and Brauner, 2010) or adrenergically-activated RBC β-NHE (Berenbrink et al., 2005), the pHe:pHi relationship observed in this study might be expected *a priori* (Brauner and Berenbrink, 2007), and is consistent with that observed in the armoured catfish, which also lacks RBC β-NHE (Brauner et al., 2004).

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., Wood et al., 1990; Wood and LeMoigne, 1991). 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 (Gesser and Poupa, 1978)]. Contrary to this pattern in other fishes, white sturgeon completely protected pHi in heart, liver, brain, and white muscle during a respiratory acidosis induced by hypercarbia (Fig.

3.7A). This tissue pHi protection was not attributable to measured intrinsic buffering for heart, liver, and white muscle (Fig. 3.10), as measured values were quite low, even in comparison with the CO_2 sensitive rainbow trout (Milligan and Wood, 1986; Wood et al., 1990). 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* (Pörtner, 1990; Shi et al., 1997), actual values in sturgeon would be even lower than those reported here. Consequently, cellular trans-membrane exchange of acid-base relevant ions must be responsible for pHi regulation in these tissues which would ultimately elevate intracellular HCO_3^-.

Assuming that the net intracellular HCO_3^- uptake associated with pHi regulation represents uptake of HCO_3^- from the environmental water (presumably in exchange for Cl^-) a simple calculation can provide insight into the rate of HCO_3^-/Cl^- exchange that would be required. Assuming sturgeon extracellular fluid comprised 25% of total body water and white muscle intracellular water the remaining 75%, and that pHi compensation can be accomplished within 6 h (as preliminary studies suggest), the rate of net acid excretion (expressed as bicarbonate uptake) from the environment at 45 mm Hg PCO_2 over that first 6 h would be approximately 0.50 μmol HCO_3^- g^{-1} water. This value is surprisingly similar to the rate found in *P. pardalis* (0.55 μmol HCO_3^- g^{-1} water) calculated using a similar approach (Brauner et al., 2004). While further investigation is necessary to verify this calculation, it is well within the capacity for net HCO_3^-/Cl^- exchange (Brauner et al., 2004).

The relationship between pHe 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 pHe and tissue pHi (Wood et al., 1990; Wood and LeMoigne, 1991). Furthermore, this study suggests that, under certain conditions, pHi can be elevated above normocapnic levels (Figs. 3.1B,C, 3.9) during a blood acidosis. Because pHi is closer to the equilibrium constant (pK) for the hydration of CO_2, much smaller increases in

[HCO_3^-] in the intracellular relative to extracellular space are required for pH recovery during a respiratory acidosis (Table 3.2). While preferentially regulating pHi over whole body pH may represent a reduced ionoregulatory cost at lower CO_2 tensions, it is possibly the only feasible option for survival when PCO_2 exceeds 15 mm Hg, the apparent limitation to pHe compensation observed in fishes (Chapter 1; Heisler, 1999; Brauner and Baker, 2009).

While some protective responses have been observed in response to an acidosis in fish tissues and cells (e.g., down regulation of protein synthesis, Langenbuch and Pörtner, 2003), 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 pHi regulatory response (Brauner et al., 2004) of the magnitude seen in this study, although *S. marmoratus* was able to protect cardiac and white muscle pH during the much less severe respiratory acidosis associated with hypoxia induced air breathing (Heisler, 1982). 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 (Poupa and Johansen, 1975; Graham et al., 1990; Krumschnabel et al., 2001; Langenbuch and Pörtner, 2003). For example, rainbow trout hepatocytes have both NHE and Na^+/HCO_3^- co-transporters that contribute to pHi regulation during acid loading (Furimsky et al., 2000). In addition, NHE activity is higher at low O_2 levels, suggesting a role in correcting for anoxia-induced acidosis (Tuominen et al., 2003). 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 (Krumschnabel et al., 2001). Under conditions of hypercarbia, when plasma HCO_3^- levels are elevated 2 to 10-fold over normal levels, Cl^-/HCO_3^- exchange may be more favoured energetically – these energetic savings could be associated with observations of over-

compensatory 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.

3.5.4 Conclusions

Clearly, white sturgeon have the capacity to alter net epithelial acid-base relevant ion transport to drive pHe recovery during moderate levels of hypercarbia (11.5 mm Hg PCO_2), despite previous evidence to the contrary (Crocker and Cech, 1998). 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 brief (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 (Chapter 1; Brauner and Baker, 2009) or elasmobranchs (Heisler et al., 1988; Wood et al., 1990). 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 (Chapter 1; Brauner and Baker, 2009), and high CO_2 tolerance has been observed in some elasmobranchs in the absence of pHi protection (Hayashi et al., 2004). As sturgeon represent the most basal osmoregulating fishes 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 fishes.

3.6 Tables

Table 3.1 The effect of short-term (6, 24, and 48 h) hypercarbia (11.5 mm Hg PCO_2) on plasma ion status and blood glucose in white sturgeon. Values are means ± s.e.m. Dissimilar letters signify discrete subsets, and thus letters indicate significant difference among treatments.

		Duration of exposure to 11.5 mm Hg PCO_2		
	Control	6 h	24 h	48 h
Plasma $[Cl^-]$ (mmol l^{-1})	121 ± 1[a]	114 ± 2[b]	105 ± 2[c]	101 ± 2[c]
Plasma $[Na^+]$ (mmol l^{-1})	136 ± 2	138 ± 1	136 ± 1	134 ± 2
Plasma $[Mg^{2+}]$ (mmol l^{-1})	0.93 ± 0.08	1.00 ± 0.04	0.99 ± 0.03	0.95 ± 0.04
Plasma $[Ca^{2+}]$ (mmol l^{-1})	2.05 ± 0.03[a]	2.23 ± 0.05[b]	2.35 ± 0.05[b]	2.20 ± 0.07[b]
Plasma osmolarity (mOsm l^{-1})	265 ± 3	266 ± 5	263 ± 2	258 ± 3
Plasma glucose (mmol l^{-1})	–[1]	7.73 ± 1.63[a]	4.49 ± 0.38[ab]	3.40 ± 0.32[b]

1. not determined.

Table 3.2 The effect of short-term (48 h) hypercarbia (Series 1, 11.5 mm Hg PCO_2; Series 2, 22.5 and 45 mm Hg PCO_2) on tissue intracellular [HCO_3^-] in white sturgeon. Values are means ± s.e.m. An asterisk indicates significant difference from respective control treatment.

	Series 1		Series 2		
	Normocarbia	11.5 mm Hg PCO_2	Normocarbia	22.5 mm Hg PCO_2	45 mm Hg PCO_2
	48 h	48 h	48 h	48 h	48 h
Red blood cell (mmol l^{-1})	1.9 ± 0.3	11.5 ± 1.0*	1.9 ± 0.2	8.3 ± 1.3*	13.2 ± 0.8*
Brain (mmol l^{-1})	1.3 ± 0.2	12.4 ± 1.3*	1.4 ± 0.4	6.9 ± 0.03*	19.7 ± 2.0*
Heart (mmol l^{-1})	1.1 ± 0.1	7.7 ± 0.3*	0.8 ± 0.06	7.1 ± 1.8*	14.3 ± 1.5*
Liver (mmol l^{-1})	0.8 ± 0.03	5.7 ± 0.2*	0.6 ± 0.13	6.9 ± 0.1*	10.7 ± 1.1*
White muscle (mmol l^{-1})	1.95 ± 0.2	6.1 ± 0.5*	1.1 ± 0.13	6.9 ± 0.1*	17.6 ± 2.2*

3.7 Figures

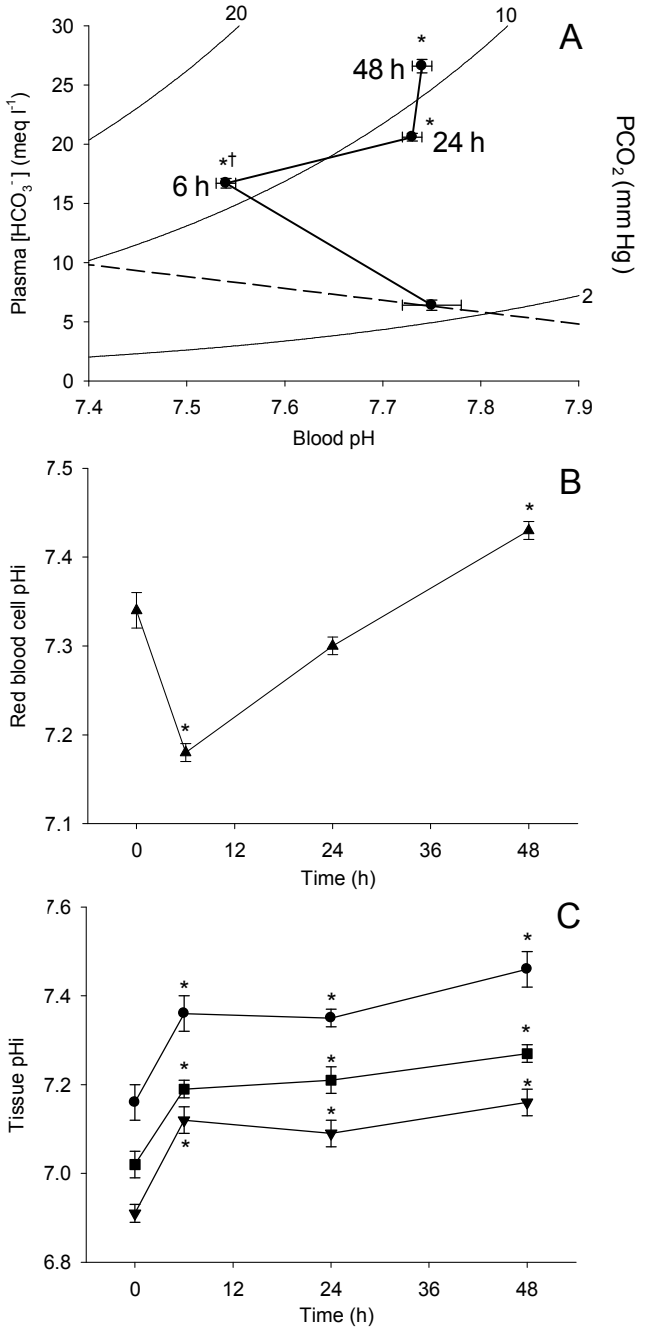


Figure 3.1 The effect of short-term (6, 24, and 48 h) hypercarbia (11.5 mm Hg PCO_2) on A) arterial pH and plasma [HCO_3^-] (mmol l^{-1}) presented as a pH/HCO_3^-/CO_2 diagram, B) red blood cell (RBC) pHi, and C) brain (circle), liver (triangle), and heart (square) pHi in white sturgeon, *A. transmontanus*. Values (n=6-7) are presented as means ± s.e.m. In A), time (h) is indicated next to each point, the dotted line represents the blood non-bicarbonate buffer line, a dagger indicates a significant change in pH, and an asterisk indicates significant change in plasma [HCO_3^-] from normocarbia (control). In B) and C), an asterisk indicates a significant difference from normocarbia (control).

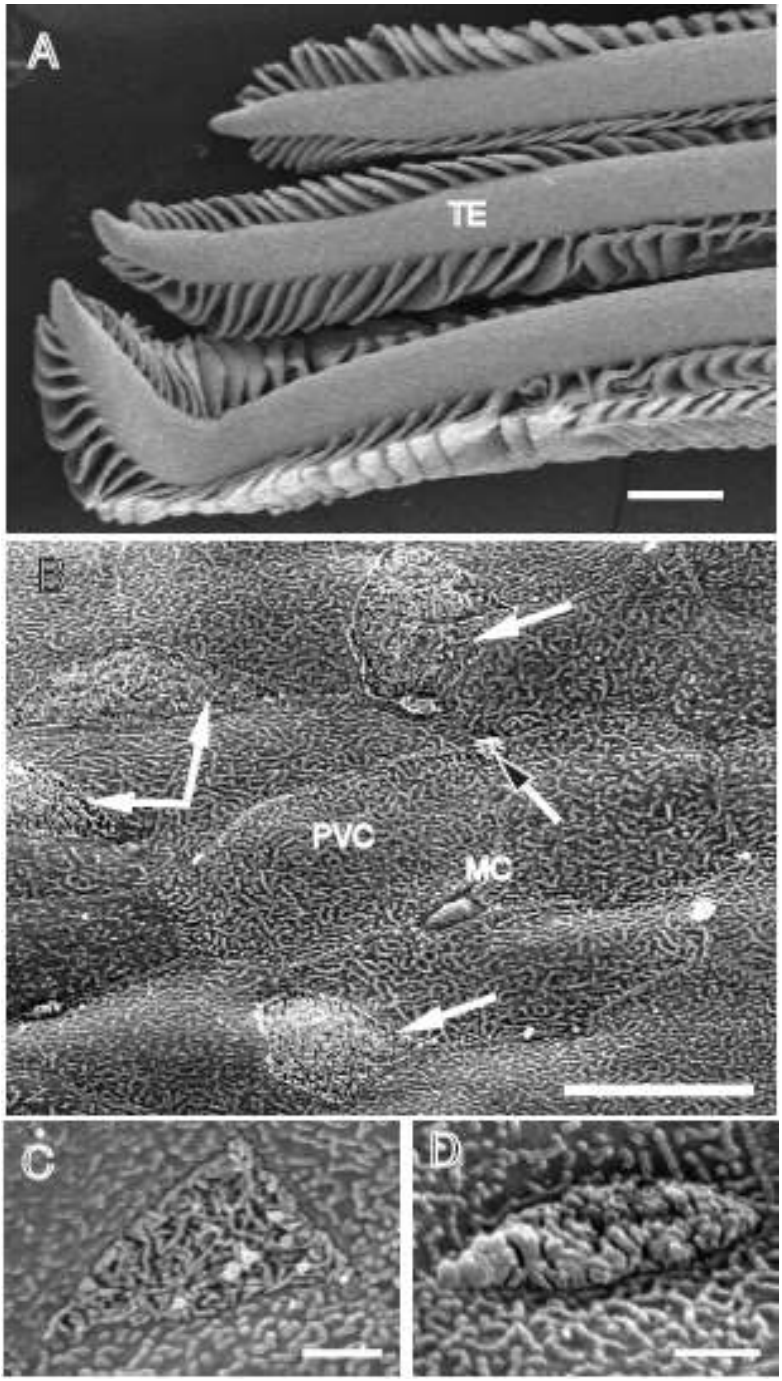


Figure 3.2 Microstructure of A) the epithelium covering the trailing edge (TE) of gill filaments (scale bar: 100 μm), B) pavement cells (PVC), mucous cells (MC), and mitochondrial-rich cells with large apical surface area (MRCLA, white arrows) and smaller surface area (MRCSA, blackhead arrows) (scale bar: 10 μm), C) long and thin microvilli representative of MRCLA (scale bar: 2 μm) and D) short and thick microvilli representative of MRCSA (scale bar: 2 μm) on the surface of gill filament epithelium in white sturgeon exposed to normocarbia.

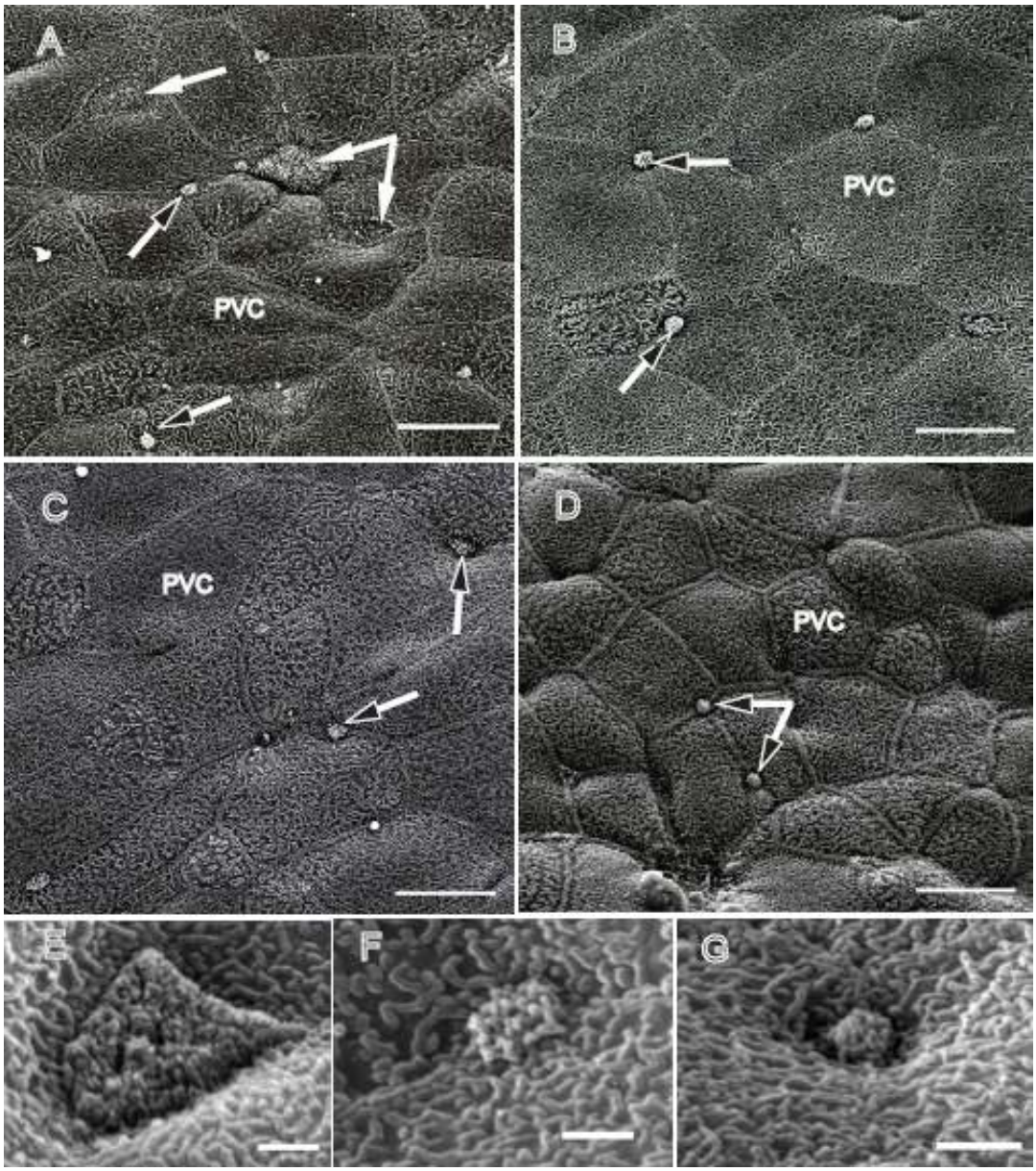


Figure 3.3 Ultrastructure of filament epithelium in gills of white sturgeon following exposure to A) normocarbia for 48 h, or moderate hypercarbia (11.5 mm Hg PCO_2) for B) 6 h, C) 24 h, and D) 48 h. MRCLA are indicated with whitehead arrows (note absence in B-D), MRCSA with blackhead arrows (scale bars: 5 µm). Apical ultrastructure of MRCSA during exposure to E) normocarbia for 48 h, and hypercarbia (11.5 mm Hg PCO_2) for F) 24 h and G) 48 h under greater magnification (scale bars: 1µm).

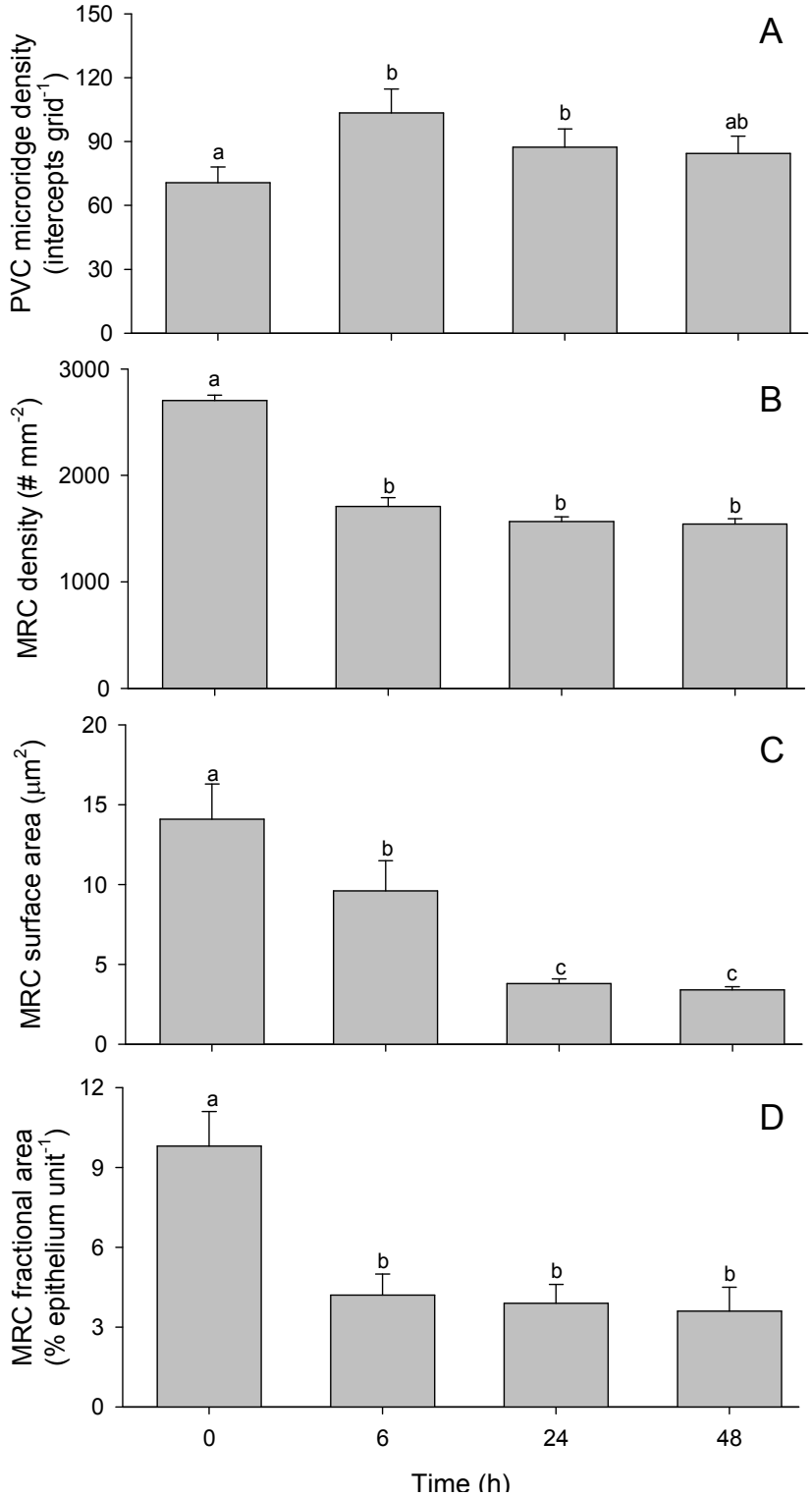


Figure 3.4 The effect of short-term (6, 24, and 48 h) moderate hypercarbia (11.5 mm Hg PCO_2) on A) pavement cell microridge density (intercepts $grid^{-1}$), B) mitochondrial-rich cell (MRC) density (number mm^{-2}), C) MRC surface area (μm^2), and D) MRC fractional area (FAMRC; % epithelium $unit^{-1}$) on the filament epithelium in the white sturgeon. Values are presented as means ± s.e.m. (n = 6-7). Letters indicate significant differences between groups.

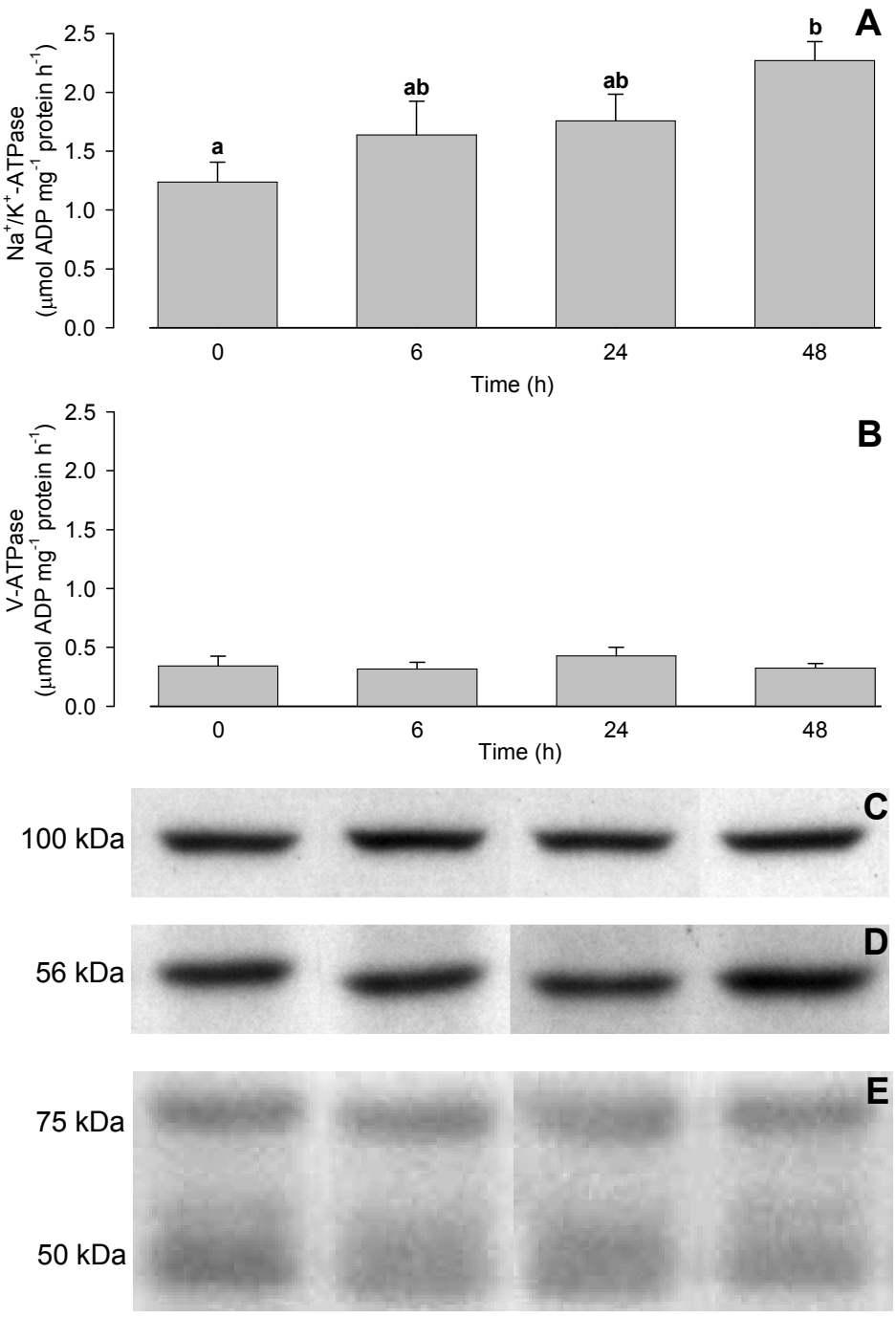


Figure 3.5 The effect of short-term (6, 24, and 48 h) moderate hypercarbia (11.5 mm Hg PCO_2) on activity of either A) branchial Na^+,K^+-ATPase (NKA) activity (µmol ADP mg^{-1} $protein^{-1}$), B) V-ATPases activity (µmol ADP mg^{-1} $protein^{-1}$), or expression of C) α subunit of NKA, D) B subunit of V-ATPase, or E) NHE3 in representative western blots in white sturgeon. In A and B, values are presented as means ± s.e.m. (n=6-7). Letters indicate significant differences between groups.

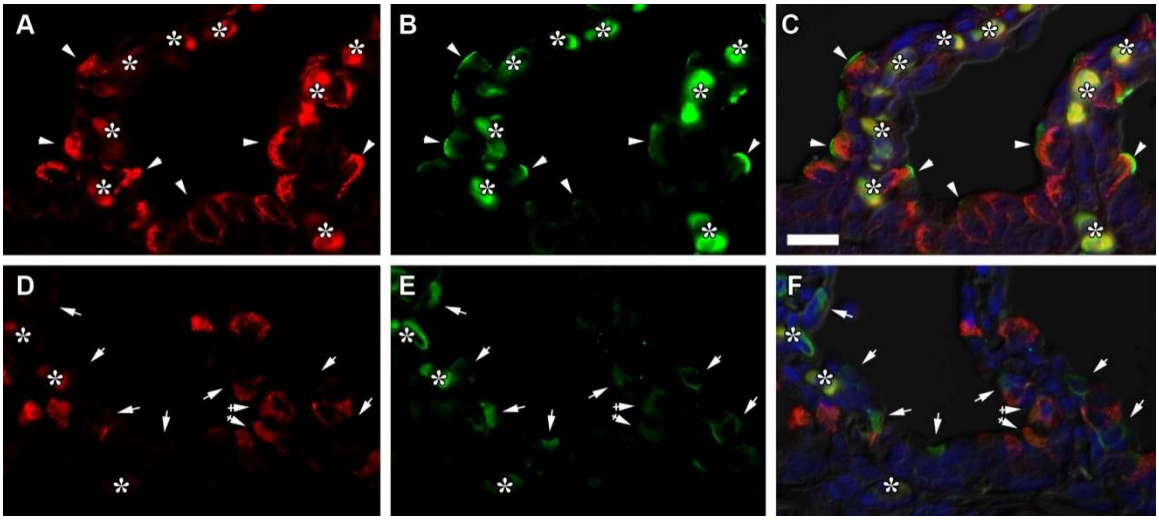


Figure 3.6 Indirect immunofluorescent localization of Na^+,K^+-ATPase α subunit (A, D) with either (B) NHE3 or V-ATPase B subunit (E) in normocarbic sturgeon gill sections (scale bar: 20 μm). Merged images of counter stained (DAPI, blue) sections were overlaid for tissue orientation (C, F). Arrowheads (A-C) indicate NHE3 immunoreactive (IR) cells, arrows (D-F) indicate V-ATPase IR cells, crossed arrows (D-F) indicate cells that double label with V-ATPase and Na^+,K^+-ATPase, and asterisks indicate erythrocytes. Moderate hypercarbia (11.5 mm Hg PCO_2 for 48 h) did not qualitatively alter the staining patterns of either NHE3 or V-ATPase (data not shown).

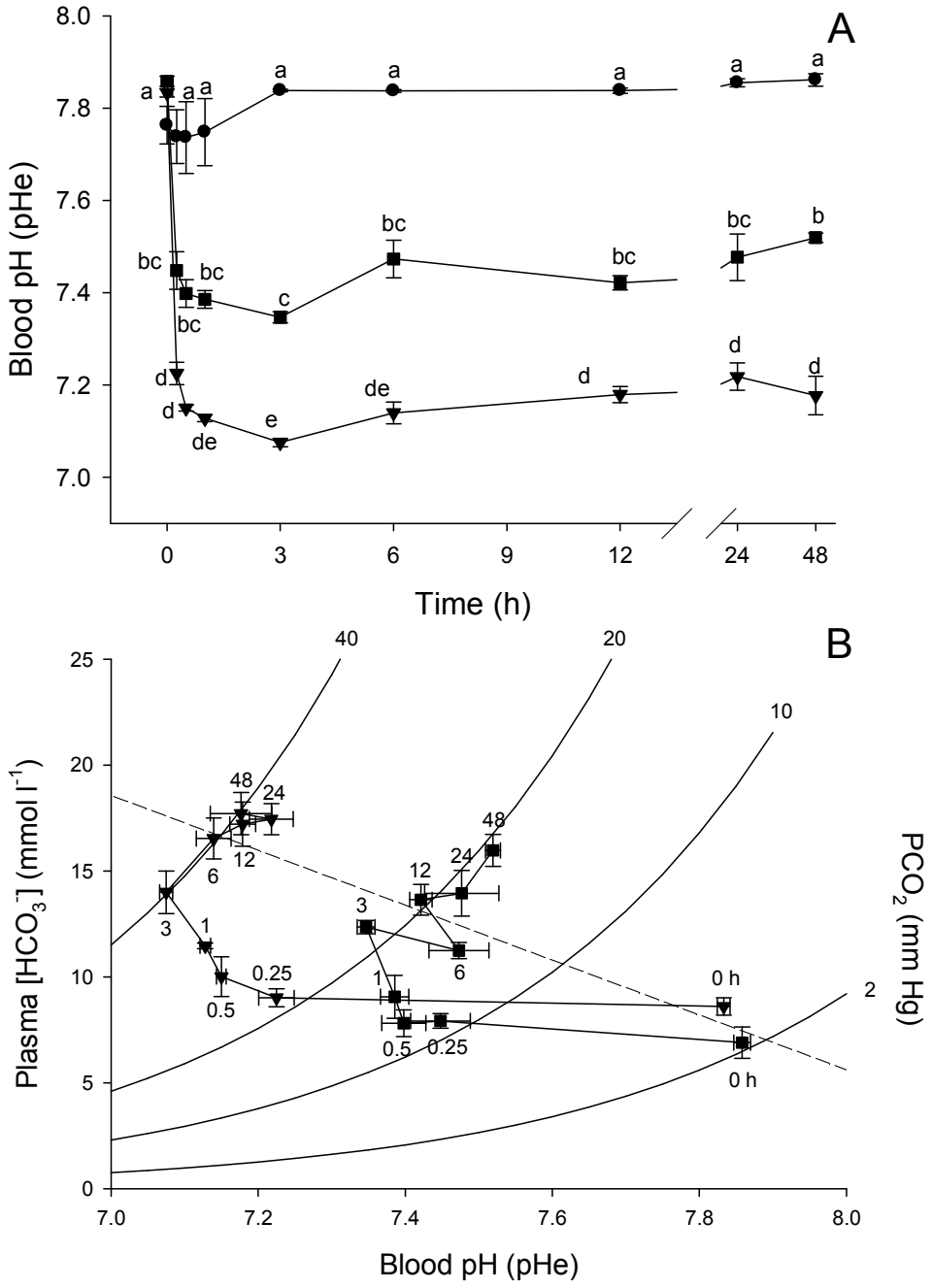


Figure 3.7 The effect of short-term (48 h) severe hypercarbia (normocarbia, circles; 22.5 mm Hg PCO_2 squares; or 45 mm Hg PCO_2 , inverted triangles) on blood pH and plasma [HCO_3^-] in cannulated white sturgeon. Blood pH is plotted as A) a function of time, sampled at 15 and 30 minutes, and 1, 3, 6, 12, 24 and 48 h, and B) against plasma [HCO_3^-], represented on a pH/HCO_3^-/CO_2 plot. Values are presented as means ± s.e.m. (n=4-6). In A), letters indicate differences between groups. In B), numbers on figure indicate time in hours, and dotted line indicates intrinsic buffer line for blood oriented through normocarbic data (normocarbic data presented in A, not shown in B for clarity).

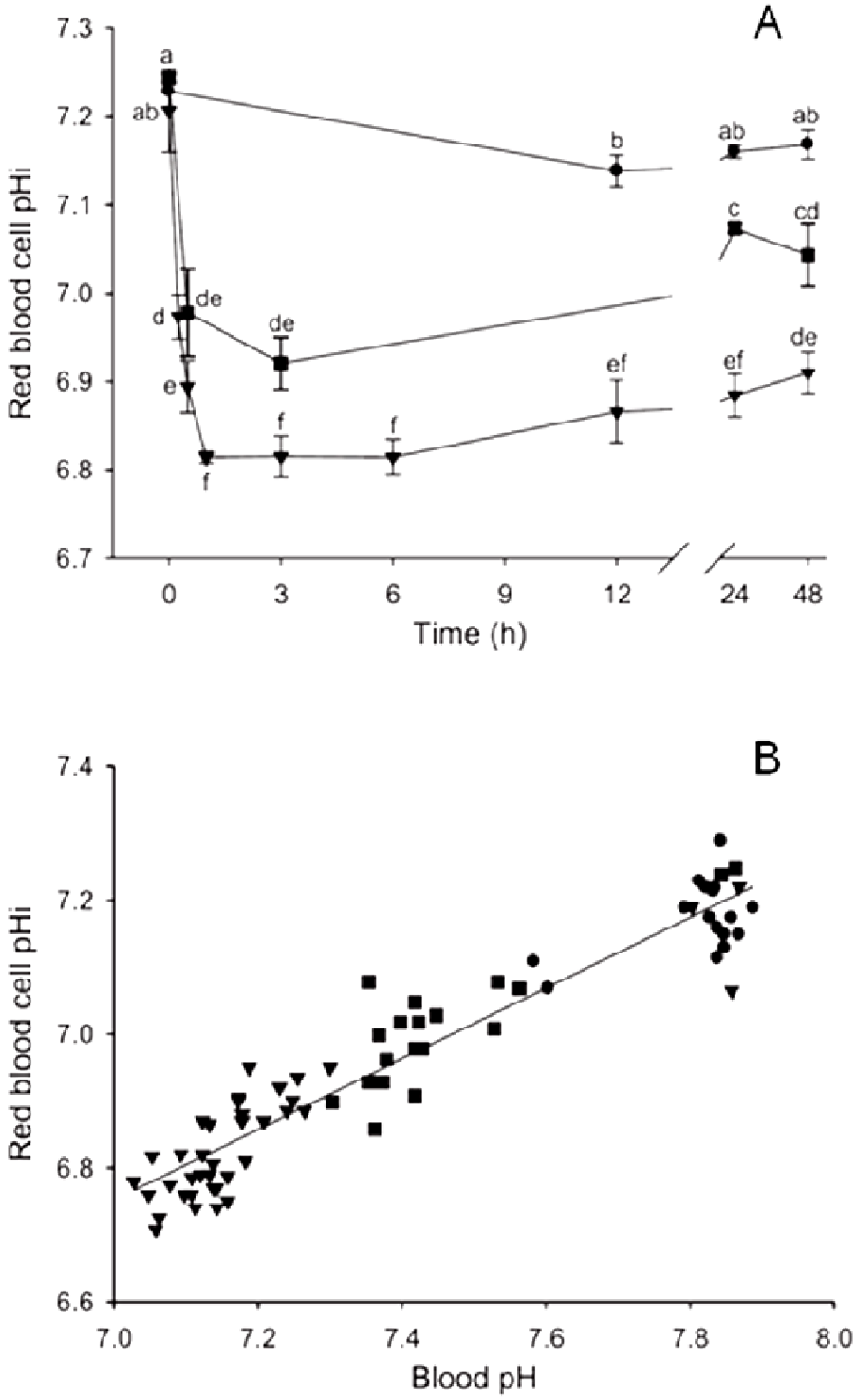


Figure 3.8 The effect of short-term (48 h) severe hypercarbia (normocarbia, circles; 22.5 mm Hg PCO_2 squares; or 45 mm Hg PCO_2 , inverted triangles) on red blood cell (RBC) intracellular pH (pHi) as a function of A) time or B) blood pH in cannulated white sturgeon. In A), values are presented as means ± s.e.m. (only groups where n > 3 are presented), and different letters indicate time points within a treatment that are significantly different. In B), the correlation between blood pH (pHe) and RBC pHi was significant (slope = 0.52, $r^2 = 0.90$, $p < 0.05$).

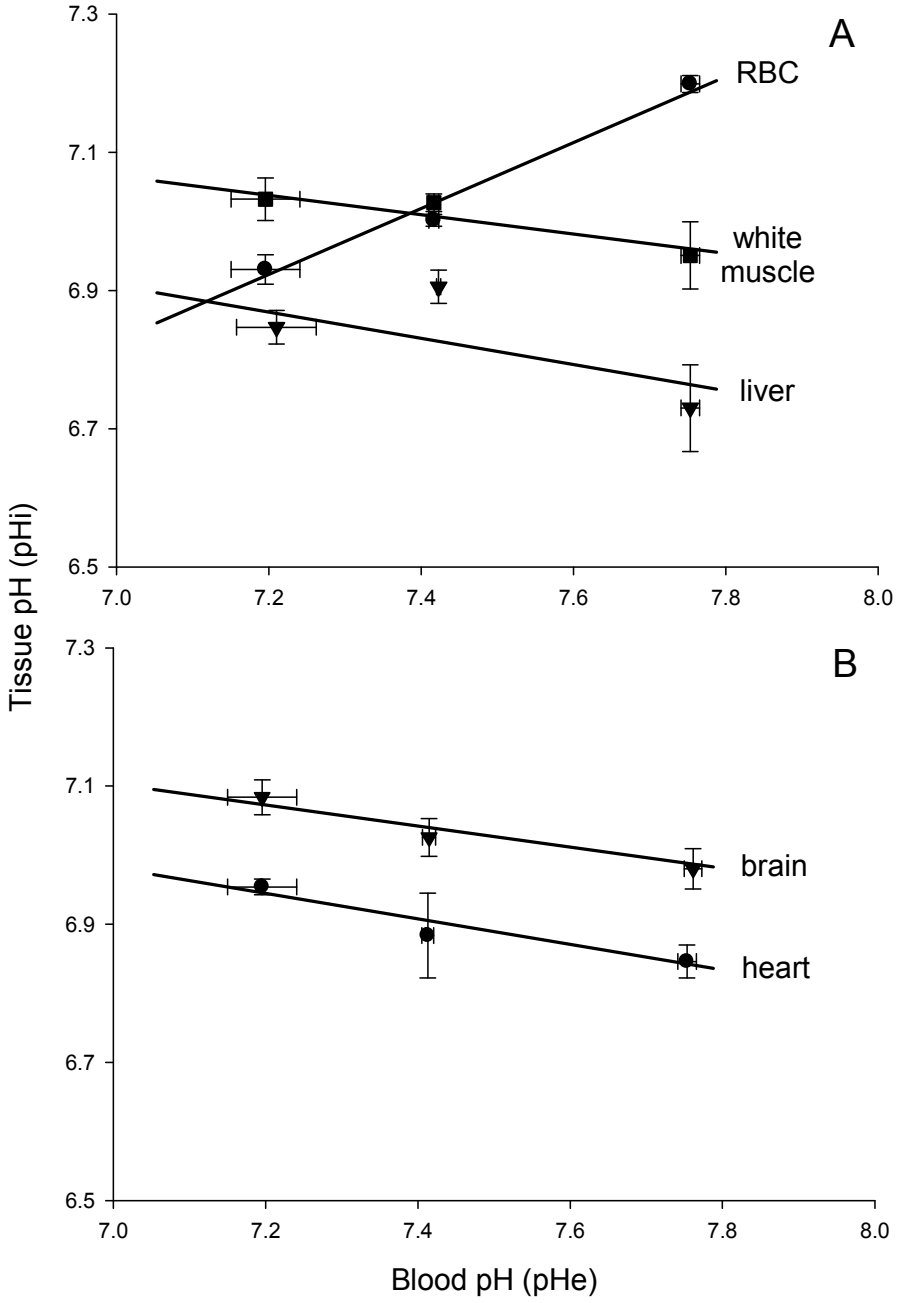


Figure 3.9 Relationship between blood extracellular pH (pHe) and intracellular pH (pHi) of RBC (circles), white muscle (squares) and liver (inverted triangle) (A) and heart (circles) and brain (inverted triangles) (B) of white sturgeon following 48 h of exposure to either normocarbia (air-equilibrated water) or severe hypercarbia (22.5 and 45 mm Hg PCO_2). Tissues are presented in separate panels for clarity. Values are presented as means ± s.e.m. (n = 4-6). Correlations between raw data for pHe and tissue pHi are described by the following lines: RBC: m = 0.48, r^2 = 0.96, $P < 0.05$; heart: slope 0.14, $r^2 = 0.67$, $P < 0.05$; brain: slope = 0.24, $r^2 = 0.72$, $P < 0.05$; 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).

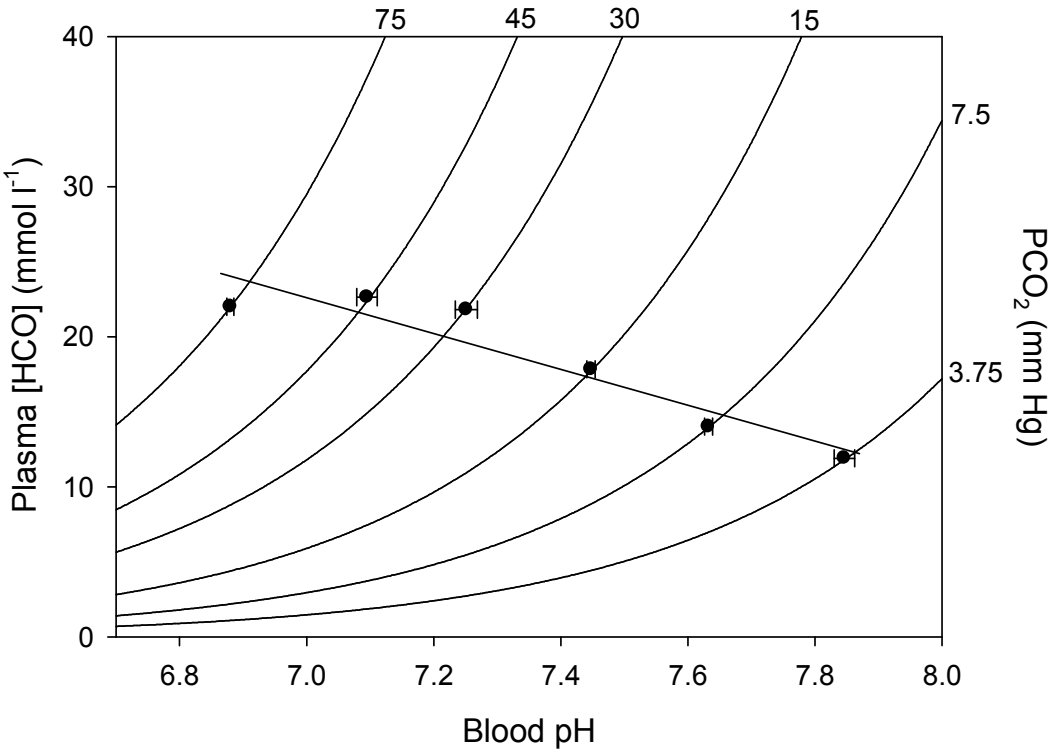


Figure 3.10 Relationship between blood pH (pHe) and plasma [HCO_3^-] in blood equilibrated *in vitro* at 3.75, 7.5, 15, 30, 45, and 75 mm Hg PCO_2. Values are means ± s.e.m. (n = 4). Intrinsic buffer capacity of blood (β_{NB} = -11.9 mmol HCO_3^- mmol l^{-1} pH $unit^{-1}$, r^2 = 0.878) was calculated from the slope of the best-fit linear regression over *in vivo* pHi values.

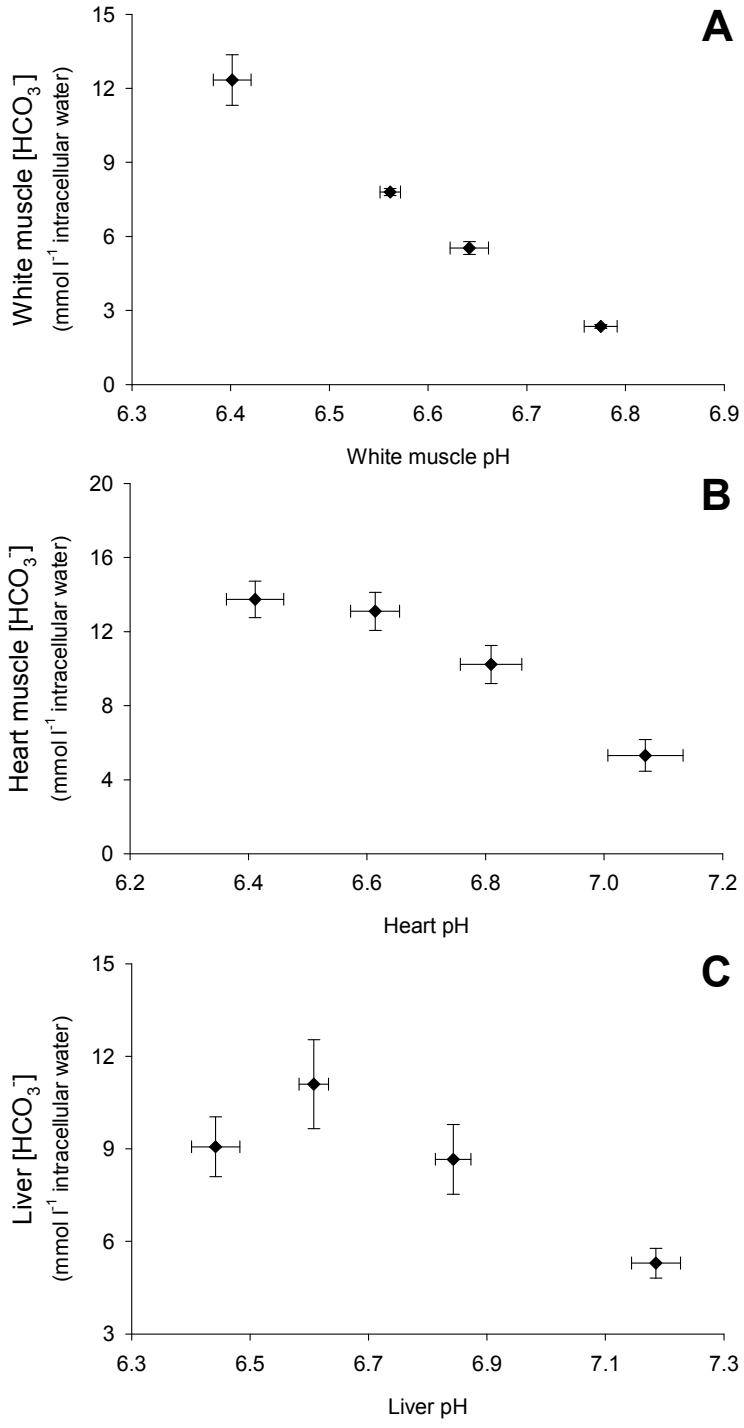


Figure 3.11 Relationship between pH and $[HCO_3^-]$ in tissue homogenates prepared from white muscle (A), heart (B), and liver (C), equilibrated at 3.75, 7.5, 15, and 30 mm Hg PCO_2. Values are means ± s.e.m. (n = 6-8). Intrinsic buffering of these homogenates (β_{NB}) were calculated from the slope of the best-fit regression (white muscle, $r^2 = 0.85$; heart, $r^2 = 0.78$; liver, $r^2 = 0.13$) over *in vivo* relevant pHi values, and tissue buffer capacity was calculated from these values (see text for details).

4: METABOLIC EFFECTS OF AQUATIC HYPERCARBIA

4.1 Synopsis

As discussed in Chapter 3, white sturgeon, *Acipenser transmontanus*, exhibit preferential pHi regulation in tissues such as the heart and brain during aquatic hypercarbia. To provide insight into potential metabolic costs associated with preferential pHi regulation, here I investigate metabolic changes concurrent with hypercarbia exposure. White sturgeon were exposed to hypercarbia (ambient, 15, 30, 45 and 60 mm Hg PCO_2), and oxygen consumption rate and a suite of organismal (e.g., activity level) and biochemical (e.g., rate of protein synthesis) parameters associated with changes in metabolic rate were measured. White sturgeon exhibited no morbidity following a 96-h exposure to 45 mm Hg $PwCO_2$, confirming them to be one of the most CO_2-tolerant fish species studied to date. Severe hypercarbia (≥ 45 mm Hg PCO_2) elicited an uncompensated acidosis in blood (~1.0 pH units) and red blood cells (~0.5 pH units), but small increases in liver (~0.25 pH units) and white muscle (~0.1 pH units) pHi after 6 h. White sturgeon $\dot{M}_{O_2}$ increased during exposure to 15 and 30 mm Hg PCO_2, but decreased at higher CO_2 tensions (45 and 60 mm Hg PCO_2). Complete pHi compensation occurred concomitantly with a decrease in rate of oxygen consumption during severe (≥ 45 mm Hg PCO_2) aquatic hypercarbia. In contrast, blood pH (pHe) compensation when observed (at 15 and 30 mm Hg PCO_2) was loosely associated with an increased rate of oxygen consumption. In this study, preferential pHi regulation occurred in the absence of an increase in whole animal metabolism, a finding which supports current hypotheses regarding the origin of this strategy of hypercarbia tolerance.

4.2 Introduction

In fish, aquatic hypercarbia induces a rapid acidosis (Chapter 1), and is typically associated with a suite of behavioural, respiratory (e.g. ventilatory response, Milsom, 2002), physiological (e.g., acid-base regulatory response, Heisler, 1999) and morphological (e.g., branchial chloride cell surface area, Perry and Gilmour, 2006) responses. These responses include pHe compensation for a respiratory acidosis induced by low to moderate hypercarbia (<15 mm Hg PCO_2), but exposure to higher CO_2 levels (> 15 mm Hg) induces a respiratory acidosis beyond the capacity for extracellular pH (pHe) compensation. Therefore, most fishes (e.g., rainbow trout, *Oncorhynchus mykiss*) directly transferred to CO_2 tensions above this threshold do not survive (e.g., Hayashi et al., 2004), likely as a consequence of an uncompensated acidosis.

Some fishes can tolerate CO_2 tensions far beyond this apparent threshold. This includes white sturgeon, *Acipenser transmontanus*. White sturgeon exhibit complete pHe recovery at lower CO_2 tensions as do most teleosts (11.5 mm Hg PCO_2; Baker et al., 2009a; Chapter 3), and can survive CO_2 tensions of 30 mm Hg for days despite blood pH remaining as low as 7.15 (Crocker and Cech, 1998). This CO_2 tolerance is associated with an exceptional capacity for pHi regulation (termed preferential pHi regulation) in critical tissues (such as heart, brain, muscle and liver) during both moderate (11.5 mm Hg PCO_2) and severe (22.5 and 45 mm Hg PCO_2) hypercarbia (Chapter 3; Baker et al., 2009a). However, whether preferential pHi regulation during hypercarbia imparts a significant metabolic cost in sturgeon remains unknown.

Few studies have addressed the relationship between metabolic rate and acid-base physiological responses to moderate or severe hypercarbia in CO_2-tolerant fishes. Previous work suggests that CO_2-sensitive species exhibit an increased rate of oxygen consumption ($\dot{M}_{O_2}$) in response to small increases in water PCO_2 (e.g., 5 mm Hg PCO_2, *O. mykiss*, Thomas, 1983). In

contrast, CO_2-tolerant species exhibit no change in $\dot{M}_{O_2}$ during exposure to low hypercarbia (≤ 10 mm Hg PCO_2) but decrease $\dot{M}_{O_2}$ in response to moderate and severe hypercarbia (11-40 mm Hg PCO_2, Cruz-Neto and Steffensen, 1997; Deigweiher et al., 2008). The CO_2-tolerant white sturgeon, however, increased $\dot{M}_{O_2}$ during a ~20 mm Hg increase in PCO_2 (Crocker and Cech, 2002). The reasons why $\dot{M}_{O_2}$ changes during hypercarbia and how these changes relate to CO_2 tolerance are currently unknown, but may, at least in part, reflect metabolic costs associated with pH compensatory mechanisms. For example, preferential pHi regulation during hypercarbia might be associated with increased ATP demand, which could be supplied either aerobically and be associated with increased $\dot{M}_{O_2}$, or anaerobically and result in tissue lactate accumulation. Metabolic demand during hypercarbia might also be influenced by organismal aversion behaviour (e.g., increased swimming activity). Alternately, some animals decrease metabolic rate (a strategy referred to as metabolic suppression, Hochachka and Somero, 2002) to survive challenging conditions by rapidly (within 12 h) reducing activity of expensive cellular processes, such as sodium transport *via* Na^+ K^+ ATPase (NKA) and protein turnover, in some tissues (goldfish liver, Jibb and Richards, 2008). Some evidence based on calculated net acid equivalent removal necessary for pHi recovery suggests that preferential pHi regulation may not be associated with a large metabolic cost (Brauner et al. 2004; Brauner and Baker, 2009). In this chapter, I test the hypothesis that preferential pHi regulation does not require substantial increases in metabolic demand.

Therefore, the objective of this study was to investigate metabolic changes associated with hypercarbia at both the whole animal and cellular levels in white sturgeon. First, I characterized the exceptional tolerance and acid-base physiology (including the extent of pHi regulatory capacity) of white sturgeon to rapidly induced, short-term (i.e., days) hypercarbia.

Second, I measured rate of oxygen consumption (i.e., $\dot{M}_{O_2}$) associated with short-term (48 h) hypercarbia. Third, I measured a suite of metabolically relevant organismal (e.g., spontaneous activity, ventilation frequency) parameters and biochemical indices of changes in metabolic strategies (i.e., NKA activity and maximal rate of protein synthesis in the liver, and lactate levels in heart and white muscle) during short term hypercarbia. The overall goal of this study was to provide insight into potential metabolic costs associated with acid-base regulation during hypercarbia in a CO_2-tolerant fish.

4.3 Materials and methods

4.3.1 Animals and rearing conditions

Hatchery-reared, juvenile white sturgeon, *A. transmontanus*, (1 year olds) progeny of wild stock, were provided by the Upper Columbia White Sturgeon Recovery Initiative's white sturgeon hatchery in Wardner, B.C. These fish were transported to Vancouver, B. C. by tanker truck, and held in the aquatic facilities at the Department of Zoology, University of British Columbia (UBC), Vancouver, BC, for several months prior to experimentation. All animals were held in large, aerated outdoor flow-through tanks (O_2 > 90% saturation, CO_2 < 0.2 mm Hg, 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. Mortality rate was less than 0.5% $week^{-1}$ over the 3 month holding and experimental period. All experiments were performed at the same temperature as in the holding tanks and feeding was withheld 24 h prior to

experimentation. All protocols complied with the guidelines approved by the Canadian Council on Animal Care, UBC ACC protocol # A07-0080.

4.3.2 Experimental protocols

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 measured for verification before, during (at least every 8 hours) and after experimental exposures *via* a PCO_2 electrode.

4.3.2.1 Series 1: The effect of hypercarbia on survival, haematology and acid-base physiology

White sturgeon (~50-150 g) were transferred individually without air exposure directly into a darkened box (30 l each, 10 fish per box, n=4-8 replicates per CO_2 tension) plumbed into a thermostated re-circulating (flow rate ~ 3 l min^{-1}) system containing de-chlorinated Vancouver city tap water. This system was pre-equilibrated to one of seven CO_2 tensions (ambient, 15, 30, 45, 60, 75, and 90 mm Hg PCO_2). Water O_2 saturation remained above 85% 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. Fish in each box were monitored after 3, 6, 12, 24, 48, and 96 h of exposure to hypercarbia and those that exhibited no opercular movement within 1 min were more closely examined for cardiac contraction, which in white sturgeon of this size can be detected visually. Animals that exhibited

no heart beat within a 1-min interval were deemed moribund and terminally anaesthetized in water containing MS-222 (1.0 g l^{-1}). Live fish were transferred to a recovery chamber.

To determine the extent to which liver and white muscle pHi were protected with increasing CO_2, white sturgeon were exposed to ambient, 45 or 90 mm Hg PCO_2 for 6 h and then euthanized in 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), each fish was immediately transferred to a surgery table and blood (1 ml) was drawn from the caudal vein *via* a sterile lithium-heparin-rinsed (150 i.u. ml^{-1} heparin) syringe (10 ml syringe, 23G needle), and placed on ice. Following this procedure (< 1 min), fish were killed *via* spinalectomy, and then a section of liver and white muscle were surgically excised, wrapped in pre-labelled aluminum foil, and flash frozen in LN_2 for later measurements of pHi. Blood was then separated into two aliquots; from the first, blood parameters (i.e., pH, haematocrit [HCT], and haemoglobin concentration [Hb]) were measured (see below), while the remaining blood was centrifuged (3 min@10,000 rpm), and plasma was removed to measure total CO_2 (TCO_2; model 965 Analyzer; Corning) and plasma [Cl^-] (HBI model 4425000; digital chloridometer).

Haemoglobin concentration, HCT and red blood cell (RBC) mean cell haemoglobin concentration (MCHC) were determined as described previously (Chapter 3; Baker et al., 2009a). Blood pH was measured using a thermostated capillary pH electrode (model BMS 3 MK 2, Radiometer). Blood PCO_2 and plasma [HCO_3^-] were calculated from TCO_2 and pH measurements as described previously (Brauner et al., 2004), using the CO_2 solubility coefficient (αCO_2) and pK' for rainbow trout (Boutilier et al., 1984) and a reorganization of the Henderson-Hasselbach equation. This indirect method has been validated for use with tissues from fish exposed to high CO_2 tensions (Brauner et al., 2004; Chapter 2; Baker et al., 2009b). Separated RBC pellets were analyzed for pHi using the freeze-thaw method (Zeidler and Kim, 1977). Tissues were later ground under LN_2, and pHi was measured using the metabolic inhibitor tissue

homogenate method (Pörtner et al., 1990), which I have verified to be accurate in tissues exposed to higher PCO_2 tensions (Chapter 2; Baker et al., 2009b). RBC and tissue pHi was measured using the same thermostated electrode as that described above for whole blood.

4.3.2.2 Series 2: The effect of hypercarbia on oxygen consumption rate ($\dot{M}_{O_2}$)

Juvenile white sturgeon (70-120 g, n = 7-8 for each CO_2 tensions, except 60 mm Hg PCO_2 where n = 4) were transferred without air exposure to one chamber of a 4-chamber, intermittent-flow respirometry system (Loligo Systems, Hobro, Denmark) 24 h prior to $\dot{M}_{O_2}$ measurements, which preliminary experiments indicated was sufficient time to reach a stable value. Each 2.4 l chamber received aerated water (10°C) at 1 l min^{-1}. Chambers were submerged in a water bath to ensure a constant temperature (10 ± 0.5°C) over the course of the entire 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 water and water oxygenated at atmospheric levels prior to each replicate (as per Eliason et al. 2008). These chambers had a recirculation pump in order to maintain water mixing when the inflow water was off and to minimize the effect of intermittent flow on the fish.

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 of the following pre-equilibrated CO_2 tensions: 15, 30, 45 or 60 mm Hg 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.

4.3.2.3 Series 3: The effect of hypercarbia on energetically-relevant parameters; tail beat and ventilation frequency, cell-free protein synthesis rate and tissue lactate levels.

In this series, organismal [tail beat (f_T) and ventilation frequency (f_V)], physiological (blood pH and plasma bicarbonate concentration) and biochemical parameters (cell-free maximal rate of protein synthesis, maximal NKA activity, and lactate concentration) were measured. White sturgeon were individually transferred without air exposure to one of five darkened boxes (10 fish per tank) all previously equilibrated to one of ambient, 15, 30, 45, or 60 mm Hg PCO_2. Fish were monitored 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 analysed from these recordings. 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 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.

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.

An NADH-linked assay and spectrometry were used to measure 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). Homogenates were centrifuged (1 min@5,000 g) 4°C) to remove filaments and other insoluble material. The supernatant was used directly in the assay of enzyme activity. Liver samples were assayed for ATPase activity in triplicate in the presence and absence of the NKA-specific inhibitor ouabain (final concentration 1 mmol l^{-1}) using a thermostated VersaMax Microplate Reader (Molecular Devices), and the difference in the rate of NADH oxidation between the two conditions was used to calculate NKA activity. Optimal assay conditions to give maximal enzyme activity were as follows: 100 mmol l^{-1} NaCl, 20 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} imidazole, 3 mmol l^{-1} ATP, 2 mmol l^{-1} phosphoenolpyruvate, 0.2 mmol l^{-1} NADH, 4U LDH and 5 U PK, pH = 7.5. NKA activity is expressed as μmol ADP h^{-1} mg $protein^{-1}$.

Protein synthesis rates were determined following previously described methods (Rider et al., 2006). Frozen liver was homogenized at 1:5 (w/v) in ice-cold extraction buffer and then clarified by centrifugation 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, and thawed tissue extracts (0.5 ml) were filtered through these columns to remove endogenous amino acids. This filtrate was collected and analysed for total protein using the Bradford assay. 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 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-[4,5-3H]-leucine (~300 cpm $pmol^{-1}$) and incubated at 25°C for 90 min. Negative controls, where clarified extract was replaced with distilled H_2O, were assayed for each sample. 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 ~7.6 pH with potassium carbonate. Neutralized extracts were centrifuged (5 min@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).

4.3.3 Statistical analyses

All values are presented as mean ± s.e.m., with sample sizes indicated in the text and figure captions. 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 X 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, SNK or Dunnett's post-hoc tests (as appropriate) were used to determine homogeneous subsets. When assumptions were violated, ANOVA on ranks was used to verify findings. Changes in parameters measured at a single time point, such as haematology, tissue pHi, lactate, protein synthesis rate, and NKA activity, or overall, such as mean $\dot{M}_{O_2}$, were analyzed using one-way ANOVA and Dunnett's post-hoc tests (with control values from fish exposed to ambient CO_2

tension) were used to determine differences. 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).

4.4 Results

4.4.1 Series 1: The effect of hypercarbia on survival, haematology and acid-base physiology

No morbidity (i.e., 100% survival) was observed following 96 h of exposure to ambient, 15, 30, and 45 mm Hg PCO_2 (Fig. 4.1). Direct transfer to 60, 75 and 90 mm Hg PCO_2 resulted in a significant reduction in survival relative to controls as early as 24, 12 and 6 h, respectively (Fig. 4.1). In all CO_2 exposure experiments, fish that survived the 96-h challenge completely recovered after being transferred to ambient CO_2 for a further 96 h.

White sturgeon exposed to 45 or 90 mm Hg PCO_2 for 6 h exhibited a significant decrease in pHe, RBC pHi (Fig. 4.2), and plasma [Cl^-] (Table 4.1) and a significant increase in plasma [HCO_3^-] (Table 4.1) relative to control. A significant increase in liver and white muscle pHi of liver or white muscle occurred following 6 h of exposure to 45 (liver and white muscle) or 90 (liver only) mm Hg PCO_2 (Fig. 4.2). White sturgeon HCT, Hb, and MCHC were unchanged following 6 h of exposure to 45 mm Hg PCO_2 relative to ambient values, but were significantly lower at 90 mm Hg PCO_2 (Table 4.1).

4.4.2 Series 2: The effect of hypercarbia on oxygen consumption rate ($\dot{M}_{O_2}$)

In white sturgeon exposed to hypercarbia, mean $\dot{M}_{O_2}$ values pooled over the entire 48 h exposure duration were significantly higher at 30 mm Hg PCO_2 and significantly lower at both

45 and 60 mm Hg PCO_2 than those exposed to both ambient and 15 mm Hg PCO_2 (one-way ANOVA, $p < 0.01$; Fig. 4.3A). Figure 4.3B summarizes temporal effects during CO_2 exposure, and shows that significant effects of CO_2 tension on $\dot{M}_{O_2}$ were dependent on time (two-way RM ANOVA, interaction term, $p < 0.001$). $\dot{M}_{O_2}$ of white sturgeon exposed to 15 mm Hg PCO_2 was elevated between 36 h and 42 h, but did not differ significantly from ambient levels at any other time point (Fig. 4.3B). Fish exposed to 30 mm Hg 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. 4.3B). Fish exposed to 45 and 60 mm Hg PCO_2 exhibited a significant reduction in $\dot{M}_{O_2}$ relative to ambient PCO_2 exposed fish within 90 min of transfer and this reduction lasted throughout the duration of the CO_2 exposure (data not shown). A representative trace of $\dot{M}_{O_2}$ in a single fish during exposure to ambient CO_2 is presented to illustrate intra-individual variability (Fig. 4.3C).

4.4.3 Series 3: The effect of hypercarbia on energetically-relevant parameters; tail beat and ventilation frequency, cell-free protein synthesis rate and tissue lactate levels.

There was an overall effect of CO_2 treatment and time on f_T, but no significant interaction (two-way ANOVA, Fig. 4.4). 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 mm Hg PCO_2 were significantly lower than in those exposed to 15 mm Hg 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 the 48 h at 45 and 60 mm Hg PCO_2 (Fig. 4.4), but even in control fish f_T was low (~30 min^{-1}) and 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. White sturgeon f_V was significantly different between all treatments (Fig. 4.5), but with respect to controls, f_V was significantly elevated at 15 and 30 mm Hg PCO_2 (Fig. 4.5) and significantly reduced at 45 and 60 mm Hg PCO_2 (Fig. 4.5). Within each CO_2 treatment, changes in f_V observed by 3 h were rapidly induced (i.e., within 10 minutes, data not shown). In all groups exposed to elevated CO_2, pHe decreased and plasma [HCO_3^-] increased by 3 h (Fig. 4.6A,B). In fish exposed to 15 and 30 mm Hg PCO_2, pHe was significantly higher at both 24 h and 48 h compared to lowest measured value (at 6 h and 12 h respectively) within that CO_2 exposure. Also, plasma [HCO_3^-] at 15 and 30 mm Hg PCO_2 was significantly higher by 12 h or 24 h, respectively, relative to 3 h values. No significant differences were observed in pHe or HCO_3^- levels after 3 h in fish exposed to 45 or 60 mm Hg PCO_2.

Liver cell-free protein synthesis rate (as indicated by radioactive leucine incorporated into proteins produced from liver homogenates) was unaffected by 12 h of hypercarbia (overall, 1.59 ± 0.07 pmol leucine mg total protein^{-1} h^{-1}, Fig. 4.7A). In contrast, maximal liver NKA activity was significantly lower following hypercarbia exposure for 24 h at all CO_2 tensions investigated (on average, 60% lower; Fig. 4.7B). Tissue lactate concentration following hypercarbia exposure for 24 h was significantly higher in heart tissue at 30 mm Hg PCO_2 (Fig. 4.8) but significantly lower in white muscle at 45 mm Hg PCO_2 (Fig. 4.8) compared to control values.

4.5 Discussion

White sturgeon exhibited remarkable survival following rapid transfer to hypercarbic water, with 100% survival following 96 h of exposure to 45 mm Hg PCO_2, and over 50%

survival at 60 mm Hg PCO_2, confirming this species to be one of the most CO_2-tolerant water-breathing fish species studied to date. Severe hypercarbia for 24 h (45 and 90 mm Hg PCO_2) elicited a large uncompensated acidosis in blood and RBC, but pHi in liver and muscle was significantly elevated (with the exception of muscle at 90 mmHg PCO_2 which was unchanged) compared to that of fish exposed to ambient PCO_2. Oxygen consumption rate ($\dot{M}_{O_2}$) and ventilation frequency (f_V) increased slightly during moderate hypercarbia (15 and 30 mm Hg PCO_2) but decreased to levels that were below normocarbic rates at higher CO_2 tensions (45 and 60 mm Hg PCO_2). Spontaneous activity (as quantified by f_T) was reduced during hypercarbia, but not high during normocarbia. Additionally, during severe hypercarbia, preferential pHi regulation of liver and white muscle was exhibited despite significant reductions in $\dot{M}_{O_2}$ in the absence of lactate accumulation. Thus, preferential pHi regulation in white sturgeon is not associated with an increase in overall metabolic costs. Increases in $\dot{M}_{O_2}$ occurred concurrently with extracellular pH (pHe) compensation during hypercarbia, suggesting that branchially-driven pHe compensation may require increased oxygen demand.

4.5.1 Hypercarbia survival and acid base regulation

White sturgeon are exceptionally tolerant of aquatic hypercarbia. In this study, white sturgeon exhibited no morbidity following rapid transfer to 45 mm Hg PCO_2 for 96 h (Fig. 4.1). This survival was in the face of a large increase in blood and tissue PCO_2 and a severe blood acidosis (> 0.8 pH units; Fig. 4.2, 4.6). Complete survival following short-term (6-96 h) exposure to water equilibrated with 45 mm Hg PCO_2 has not been documented in any other water breathing fish, despite repeated observation in white sturgeon (this chapter; Chapter 3;

Baker et al., 2009b). Consequently, white sturgeon are one of the most CO_2-tolerant fishes studied to date.

This tolerance may be a result of the unusual acid-base regulatory response of white sturgeon to hypercarbia. Most vertebrates studied to date exhibit a qualitatively similar acidosis in the intra- and extracellular compartment during short term hypercarbia (e.g., Rothe and Heisler, 1987; Wood et al., 1990; Wood and LeMoigne, 1991). As CO_2 is thought to exert its toxicity through a general intracellular acidosis, survival has traditionally been thought to rely at least partially on pHe compensation (Chapter 1; Brauner and Baker, 2009). In fish, pHe compensation occurs *via* an increase in net bicarbonate accumulation (i.e., net acid excretion) in exchange for plasma chloride (Lloyd and White, 1967), and this exchange is mainly driven by branchial processes (Claiborne et al., 2002). This compensation, however, remains incomplete during short term, severe CO_2 challenges (> 15 mm Hg PCO_2 and ~ 0.4 pH units; Heisler, 1999), and thus an intracellular acidosis persists. As expected, white sturgeon exhibited full, partial or no pHe compensation at 15, 30 and ≥ 45 mm Hg PCO_2 respectively within 48 h, and the magnitude of blood pH recovery was closely associated with active net accumulation of extracellular (i.e., plasma) HCO_3^-. In contrast to what is found in most other fishes, white sturgeon liver and white muscle pHi were not reduced (Fig. 4.2). In fact, pHi of these tissues increased relative to normocarbic values in surviving fish, even at CO_2 tensions that induced complete morbidity within 24 h (90 mm Hg PCO_2). Thus, complete protection of pHi of critical tissues observed in sturgeon 1-2 kg (Chapter 3; Baker et al., 2009a) and confirmed here in smaller sturgeon (50-150 g) likely contributes to their exceptional survival rates during severe hypercarbia. Changes in whole animal metabolic rate as described below may provide insight into the potential cost of the acid-base regulation exhibited by white sturgeon in response to different levels of hypercarbia.

4.5.2 Oxygen consumption rate during moderate and severe hypercarbia

In white sturgeon, $\dot{M}_{O_2}$ was influenced by water CO_2 tension, but the qualitative nature depended on the degree of hypercarbia. A moderate increase in CO_2 (to 30 mm Hg PCO_2) induced an overall increase in $\dot{M}_{O_2}$ (35%), yet more severe hypercarbia (45 and 60 mm Hg PCO_2) induced decreases (~30 and 60%, respectively). In general, few studies have measured metabolic rate during hypercarbia in CO_2-tolerant fishes. However, my findings agree with those of Crocker and Cech (2002), where white sturgeon increased $\dot{M}_{O_2}$ by 30% during exposure to 20 mm Hg PCO_2. Other work has demonstrated that CO_2-sensitive species may exhibit an increased rate of oxygen consumption ($\dot{M}_{O_2}$) in response to small increases in PCO_2 (e.g., 5 mm Hg PCO_2, *O. mykiss*, Thomas, 1983). In contrast, CO_2-tolerant species typically exhibit no change in $\dot{M}_{O_2}$ response to low hypercarbia ($\leq$ 7 mm Hg PCO_2) but decrease $\dot{M}_{O_2}$ as CO_2 tension increase moderate or severe (8-40 mm Hg PCO_2) levels (Cruz-Neto and Steffensen, 1997; Deigweiher et al., 2008). In white sturgeon, clearly, metabolic demands change according to the severity of aquatic hypercarbia to which these fish are exposed.

Estimating the overall energetic cost of the physiological processes associated with organismal responses by measuring metabolic rate is subject to limitations. For example, $\dot{M}_{O_2}$ could be altered as a result of an overlaying cost of a general stress response unrelated to hypercarbia (e.g., induced by experimental protocols). $\dot{M}_{O_2}$ in control fish, however, did not change over the course of 48 h, suggesting that CO_2 induced elevations in $\dot{M}_{O_2}$ was not the result of a stress response induced by protocols outside of those related to the hypercarbic challenge itself. Additionally, decreases in $\dot{M}_{O_2}$ during severe hypercarbia could be indicative of CO_2-induced anaesthesia. However, typical CO_2 levels used for anaesthesia are very high (135 -

760 mm Hg PCO_2), and recovery following exposure to these levels only occurs if exposure durations are short (minutes). The mechanisms with which CO_2 exerts its anaesthetic effects are uncertain, but probably relates to its toxic effects (Putnam and Roos, 1997). Previous researchers (Bernier and Randall, 1998) have suggested that even in CO_2 sensitive fishes such as rainbow trout, CO_2 as an anaesthetic requires levels higher than those used in this study. To provide stronger evidence that changes in $\dot{M}_{O_2}$ are due to experimental treatments, most of the parameters in this study were measured following 12 to 48 h of hypercarbia, in addition to any other times. My findings strongly suggest that changes in energetic demands are not greatly confounded by either stress independent of elevated CO_2 exposure, or CO_2-induced anaesthesia.

Energetic demands are also unlikely to be related to the changes observed in this study of either f_T or f_V. Changes in $\dot{M}_{O_2}$ over time (Fig 4.3B) were matched qualitatively by changes in f_V (Fig. 4.5), which is not surprising considering the link between ventilatory drive and O_2 demand. In addition, changes in ventilation amplitude appeared small as determined through visual observation. Hypercarbia typically has a stimulatory effect on total ventilation in fishes (Gilmour, 2001). In general, changes in ventilation are not thought to have a large influence on whole animal metabolic rate (Skovgaard and Wang, 2004), and thus are unlikely to account for the large changes in $\dot{M}_{O_2}$ seen here. It is also unlikely that f_T has much influence on $\dot{M}_{O_2}$, as more than a doubling of f_T was observed in control fish from 3 to 48 h, but no significant changes in $\dot{M}_{O_2}$ were associated with this increase in activity. In addition, spontaneous activity was qualitatively very low, as fish exhibited no sustained or burst swimming during f_T assessment. Therefore, I believe that the reduction in f_T at higher CO_2 levels is not likely to be responsible for up to a 60% reduction in $\dot{M}_{O_2}$ at 60 mm Hg PCO_2, and that changes in $\dot{M}_{O_2}$ are informative to some degree of acid-base related responses.

Thus, elevations in $\dot{M}_{O_2}$ during moderate hypercarbia may reflect metabolic costs associated with acid-base regulation, such as the activation of branchial compensatory mechanisms. $\dot{M}_{O_2}$ increased during exposure to CO_2 tensions of 15 mm Hg (~ 40% between 30 and 42 h) and 30 mm Hg (~ 40% overall), and these increases were concurrent with increased net acid excretion, as implied by pHe compensation. I have demonstrated an increased expression of NKA following exposure to 11.5 mm Hg PCO_2 and the presence of a V-ATPase in the gills of sturgeon, both of which are likely involved in branchial acid excretion (Chapter 3; Baker et al., 2009a). Even if only a portion of the 40% increase in metabolic rate is associated with blood pHe regulation, it may indicate that compensating for an acidosis by regulating blood pH is metabolically costly.

The capacity of white sturgeon to increase whole animal metabolic rate (maximum factorial metabolic scope; maximum $\dot{M}_{O_2}$ /standard $\dot{M}_{O_2}$) can be used to put this expense in context. Calculations of factorial metabolic scope (maximum $\dot{M}_{O_2}$ during hypercarbia/ $\dot{M}_{O_2}$ during normocarbia) from $\dot{M}_{O_2}$ measured over 6 h from this study return values as high as 1.8 during hypercarbia. Previous studies on sturgeon held at similar temperatures found similar standard metabolic rates to those reported here, and maximum factorial metabolic scope has been estimated to be between 2 to 3 as measured on sturgeons swimming at maximum velocities and during forced exercise (McKinley and Power, 1992; McKenzie et al., 2001; Geist et al., 2005; McKenzie et al., 2007). Thus, $\dot{M}_{O_2}$ changes induced by these CO_2 tensions (i.e., 15 and 30 mm Hg PCO2) represent a substantial increase. Certainly, these increased energetic demands during hypercarbia, and whether they reflect the cost of acid-base regulation warrant further investigation.

4.5.3 Changes in metabolic demands during severe hypercarbia

Severe hypercarbia (≥ 45 mm Hg PCO_2) induced a significant reduction in $\dot{M}_{O_2}$ in white sturgeon. During 45 and 60 mm Hg PCO_2, overall $\dot{M}_{O_2}$ decreased significantly (~30 and 55% respectively), and intracellular [lactate] was unchanged in heart and not elevated in white muscle after 24 h (Fig. 4.8). Relatively small changes in energetic demand to fuel pHi regulation during hypercarbia fits with current speculation regarding the proposed modest increase in intracellular HCO_3^- (Brauner et al., 2004; Brauner and Baker, 2009). For example, net plasma HCO_3^- accumulation required to compensate for a hypercarbic challenge of 45 mm Hg assuming blood pH to be 7.8 would be between 100 and 150 mmol l^{-1}. In most fishes (e.g., rainbow trout), this extracellular accumulation would be required to drive pHi compensation in tissues, although the tissues themselves might only required 10-15 $mmol^{-1}$ of net HCO_3^- accumulation (assuming pHi = 7). Extracellular fluid accounts for ~25% of whole body volume in fish, and thus regulating only pH in the intracellular compartment could represent a significant savings in acid-base transport, based on the base equivalents needing transport. Therefore, my suggestion that preferential pHi regulation can occur in the absence of a substantial increase in metabolic demand supports the possibility that this strategy is metabolically less costly than complete pHe compensation.

The reduction in $\dot{M}_{O_2}$ during severe (≤ 45 mm Hg PCO_2) hypercarbia is likely not largely influenced by changes in f_T (Fig. 4.4) or f_V (Fig. 4.5) as described earlier, but could be indicative of metabolic depression at the cellular level. White sturgeon have some capacity to reduce $\dot{M}_{O_2}$, although this has only been observed in response to hypoxia (Burggren and Randall, 1978; Crocker and Cech, 1997). White sturgeon in this study however, did not exhibit cellular responses that could account for changes in $\dot{M}_{O_2}$, nor did these responses indicate

metabolic depression. Protein synthesis rates in the liver, suggested to account for 20-30% of total ATP-coupled O_2 demand (Bickler and Buck, 2007), can be decreased between 56–95% in crucian carp, *Carassius carassius* (Smith et al., 1996), Amazonian cichlids [*Astronotus ocellatus* (Lewis et al., 2007)], and goldfish, *Carassius auratus*, (Jibb and Richards, 2008) during anoxic or hypoxic challenge. In contrast, maximal protein synthesis rates in white sturgeon liver homogenates were unaltered by hypercarbia (Fig. 4.7A). This finding is consistent with *in vitro* findings, where protein synthesis rates of hepatocytes isolated from *Lepidonotothen kempi* were not reduced by elevated CO_2, but only by an intracellular acidosis (Langenbuch and Pörtner, 2003). White sturgeon do exhibit some metabolically-relevant cellular changes, as NKA activity, estimated to account for as much as 25% of resting metabolic demand, in liver homogenates was significantly reduced at all CO_2 tensions (Fig. 4.7B). Overwintering turtles can reduce whole body NKA activity during hypoxia or anoxia to reduce metabolic costs greatly (Jackson, 2000). Thus, reductions in NKA activity following 12 h of hypercarbia may result in decreased metabolic demand, although the pattern of reduction in NKA activity does not match the changes in $\dot{M}_{O_2}$ observed at this time. In general, cellular responses in white sturgeon do not match what has been found in other vertebrates exhibiting metabolic suppression.

Both blood pH compensation (Fig. 4.6A) and HCO_3^- accumulation (Fig. 4.6B) were negligible when severe ($\geq$ 45 mm Hg PCO_2) hypercarbia dropped water pH to between 4.8 and 5.5. Previous research has demonstrated that at a pH of 5.5, V-ATPase activity and associated Na^+ uptake is significantly reduced (Lin and Randall, 1993). Certainly water pH is recognized as an important factor in branchial Na^+ uptake (Parks et al., 2007, 2008), which is likely critical for acid excretion. The loss of this capacity might explain both the lack of pHe compensation, and some of the decrease in $\dot{M}_{O_2}$ at more severe CO_2 tensions. In this way, my findings in white

sturgeon exposed to severe hypercarbia support the hypothesis that net acid excretion may be relatively energetically expensive to the costs associated with preferential pHi regulation.

4.5.4 Conclusions

Preferential pHi regulation in white sturgeon tissues during hypercarbia can occur in the absence of an increase in whole animal metabolic rate (i.e., no increase in $\dot{M}_{O_2}$ or lactate accumulation) (e.g., 45 or 60 mm Hg PCO_2). In contrast, pHe compensation, which relies heavily on increased branchially-driven net acid excretion, occurs simultaneously with an increase in $\dot{M}_{O_2}$, which may reflect metabolic demand associated with increases in net acid excretion. Sturgeons have great value for studying vertebrate evolution, as they may retain pleisiomorphic traits (Cech and Doroshov, 2004). CO_2-tolerance of white sturgeon and other basal actinopterygiian fishes (such as *Amia calva*, Brauner and Baker, 2009) suggests that hypercarbia may well have played a role in the evolution of more derived fishes (Ultsch, 1996). To summarize, the results from this chapter indicate that pHi protection in white sturgeon tissues can occur in the absence of an increase in whole animal $\dot{M}_{O_2}$, and thus I hypothesize that preferential pHi regulation during short term exposure to hypercarbia may not be metabolically costly.

4.6 Tables

Table 4.1 The effect of short-term (24 h) hypercarbia (45 and 90 mm Hg PCO_2) on haematocrit (HCT, %), haemoglobin concentration (Hb; mmol l^{-1}), mean cell haemoglobin concentration (MCHC), plasma bicarbonate concentration (mmol l^{-1}), and plasma chloride concentration (mmol l^{-1}) in white sturgeon. Values are mean ± s.e.m. An asterisk indicates a significant difference from the control treatment.

PCO_2 (mm Hg)	HCT (%)	[Hb] (mmol l^{-1})	MCHC	$[HCO_3^-]$ (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*

4.7 Figures

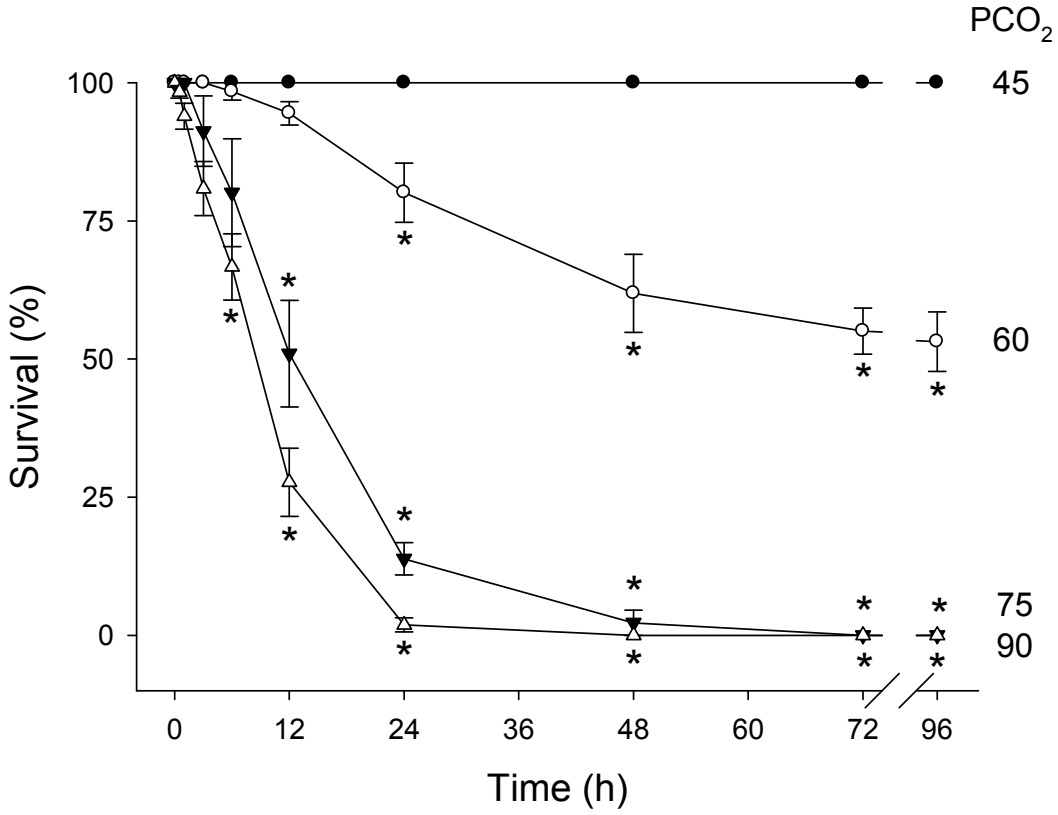


Figure 4.1 The effect of short-term (96 h) hypercarbia (45 mm Hg PCO_2, filled circles; 60 mm Hg PCO_2, open circles; 75 mm Hg PCO_2, inverted filled triangles; and 90 mm Hg PCO_2, open triangles) on white sturgeon survival (%). Values represent means ± s.e.m. (n = 4-8, with 10 fish per tank). An asterisk indicates a difference between the associated treatment and ambient PCO_2 treatment at a given sampling time. Mean survival of fish exposed to normocarbia, 15, and 30 mm Hg PCO_2 was 100% at all time points and data were removed for clarity.

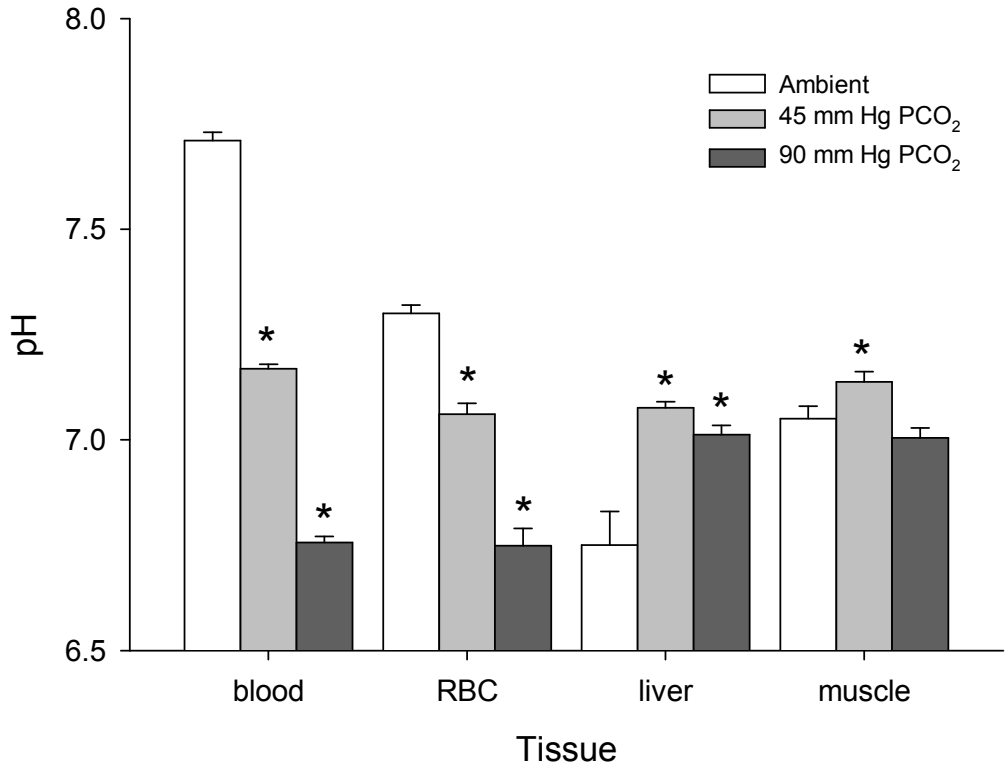


Figure 4.2 The effect of short term (6 h) hypercarbia (normocarbia, white bars; 45 mm Hg PCO_2, light bars; 90 mm Hg PCO_2 dark bars) on white sturgeon blood pH (pHe) or intracellular pH (pHi) of red blood cells (RBC), liver, and white muscle. Values are means ± s.e.m. (n = 9). An asterisk indicates a statistically significant difference from normocarbia exposed group.

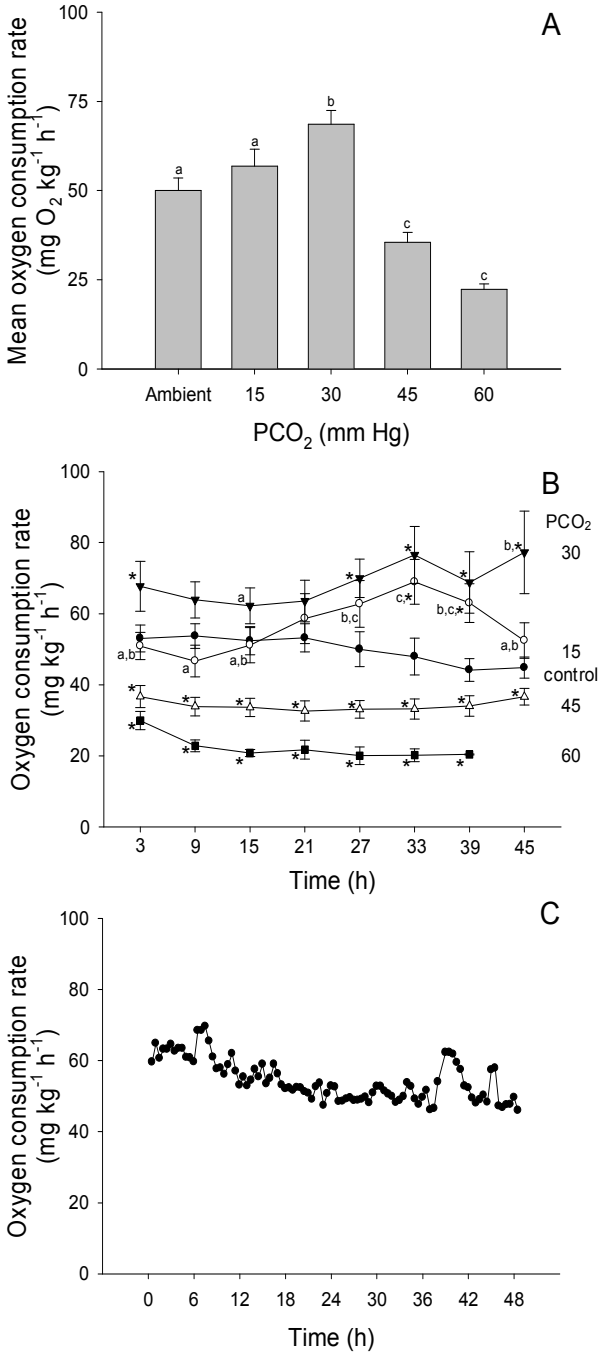


Figure 4.3 The effect of short-term (48 h) hypercarbia on A) overall mean oxygen consumption rate ($\dot{M}_{O_2}$) and B) oxygen consumption rate (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) over time (pooled in 6 h periods) of white sturgeon. Panel C shows a representative trace of a fish during normocarbia. Values are means ± s.e.m. (n = 8 for each treatment). An asterisks indicates a significant difference from normocarbic treatment at a given sampling time. Letters indicate significant differences within a treatment. At 60 mm Hg, only three fish survived 36 h, and none survived past 45 h.

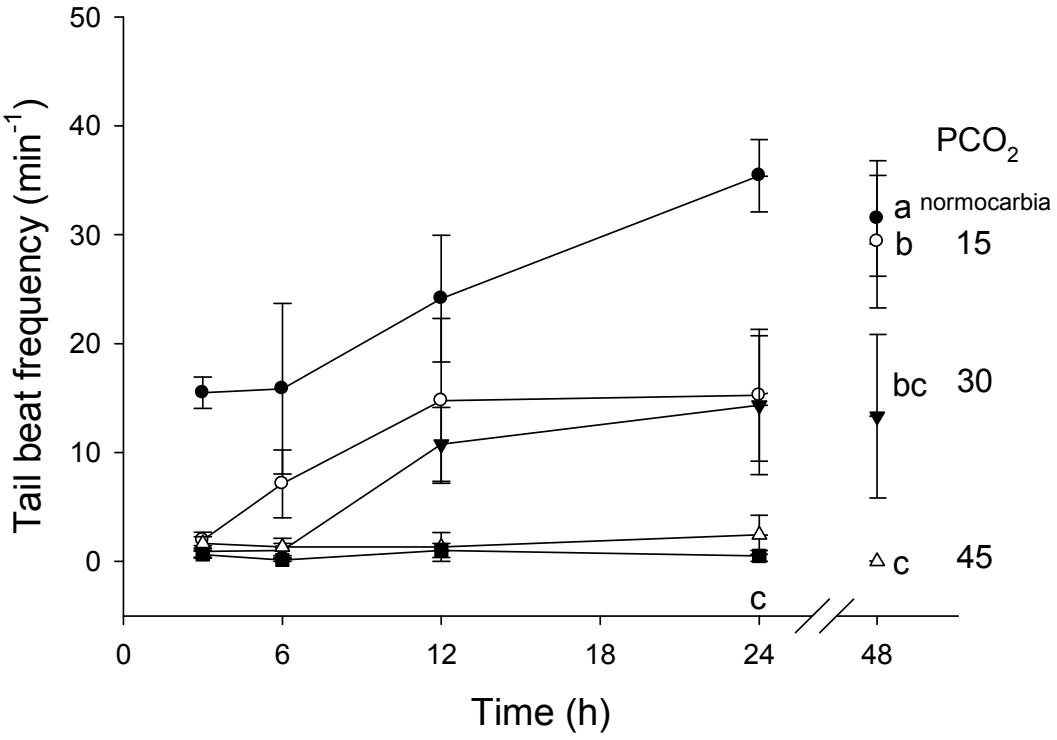


Figure 4.4 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on tail beat frequency (f_T) of white sturgeon. Values are means ± s.e.m. (n = 6-10 for each treatment). There is an overall effect of CO_2 treatment and time, but no significant interaction. Letters indicate differences between main treatment effects of CO_2. At 60 mm Hg, no fish survived 48 h.

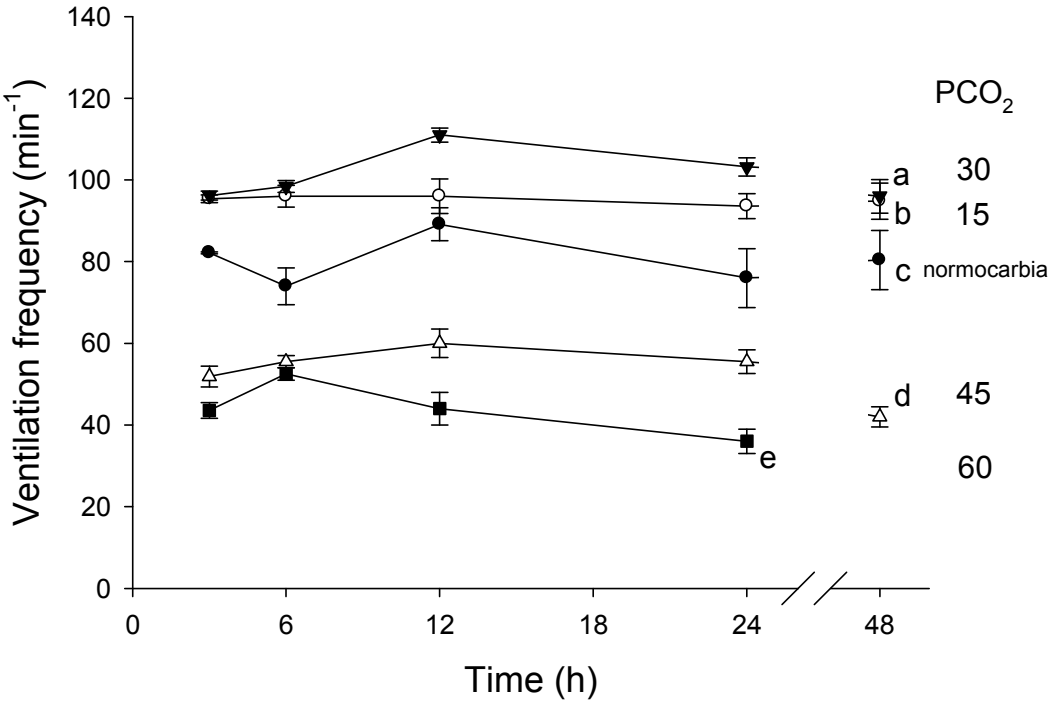


Figure 4.5 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on ventilation frequency (f_V) of white sturgeon. Values are means ± s.e.m. (n = 4-8 for each value). There is an overall effect of CO_2 treatment and time, but no significant interaction. Letters indicate differences between main treatment effects of CO_2. At 60 mm Hg, no fish survived 48 h.

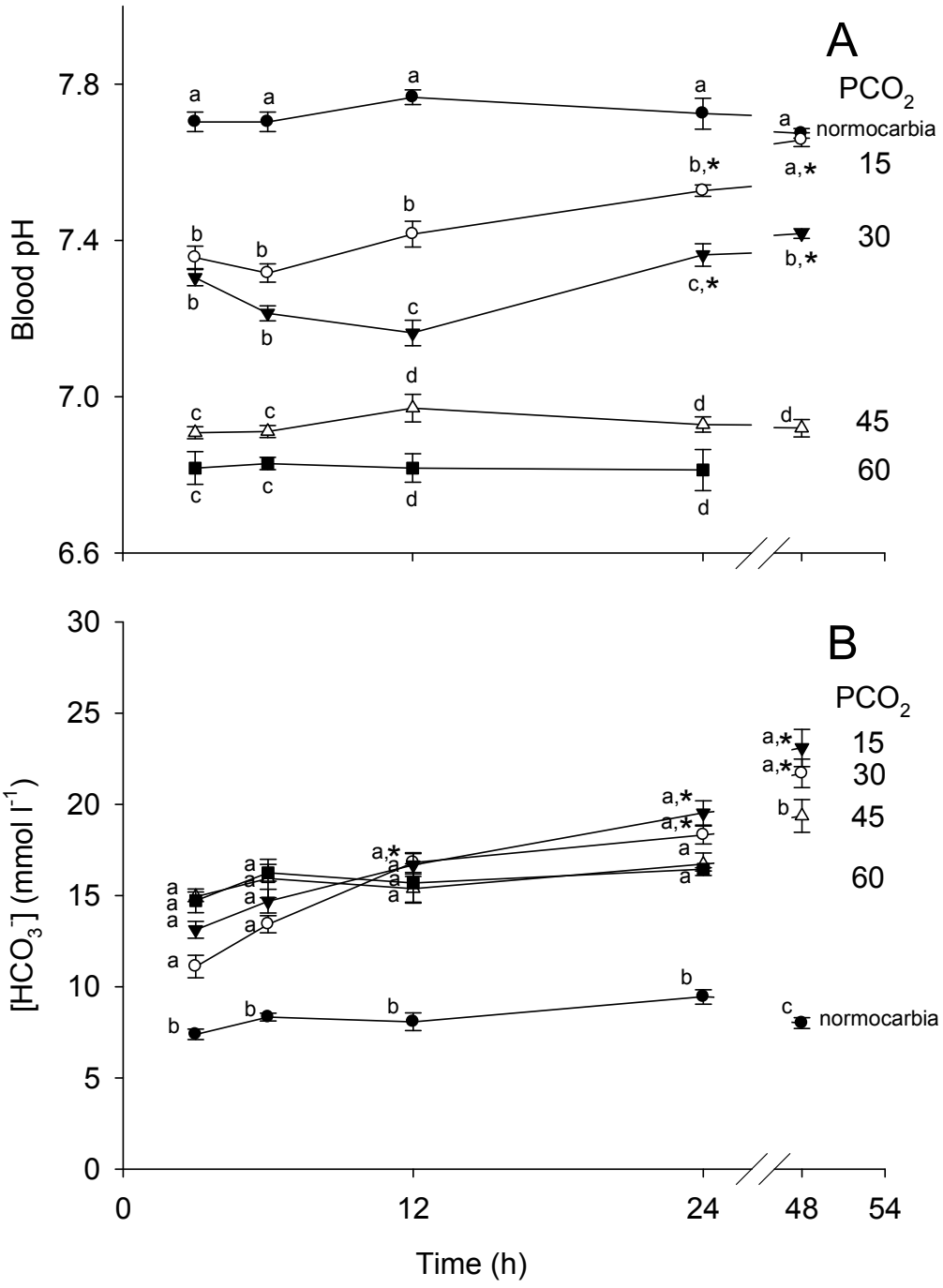


Figure 4.6 The effect of short-term (48 h) hypercarbia (normocarbia, filled circles; 15 mm Hg PCO_2, open circles; 30 mm Hg PCO_2, filled inverted triangles; 45 mm Hg PCO_2, open triangles; and 60 mm Hg PCO_2, filled squares) on A) whole blood pH and B) plasma $[HCO_3^-]$ of white sturgeon. Values represent mean ± s.e.m. (n=4-7 per group). Letters 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 mm Hg, no fish survived 48 h.

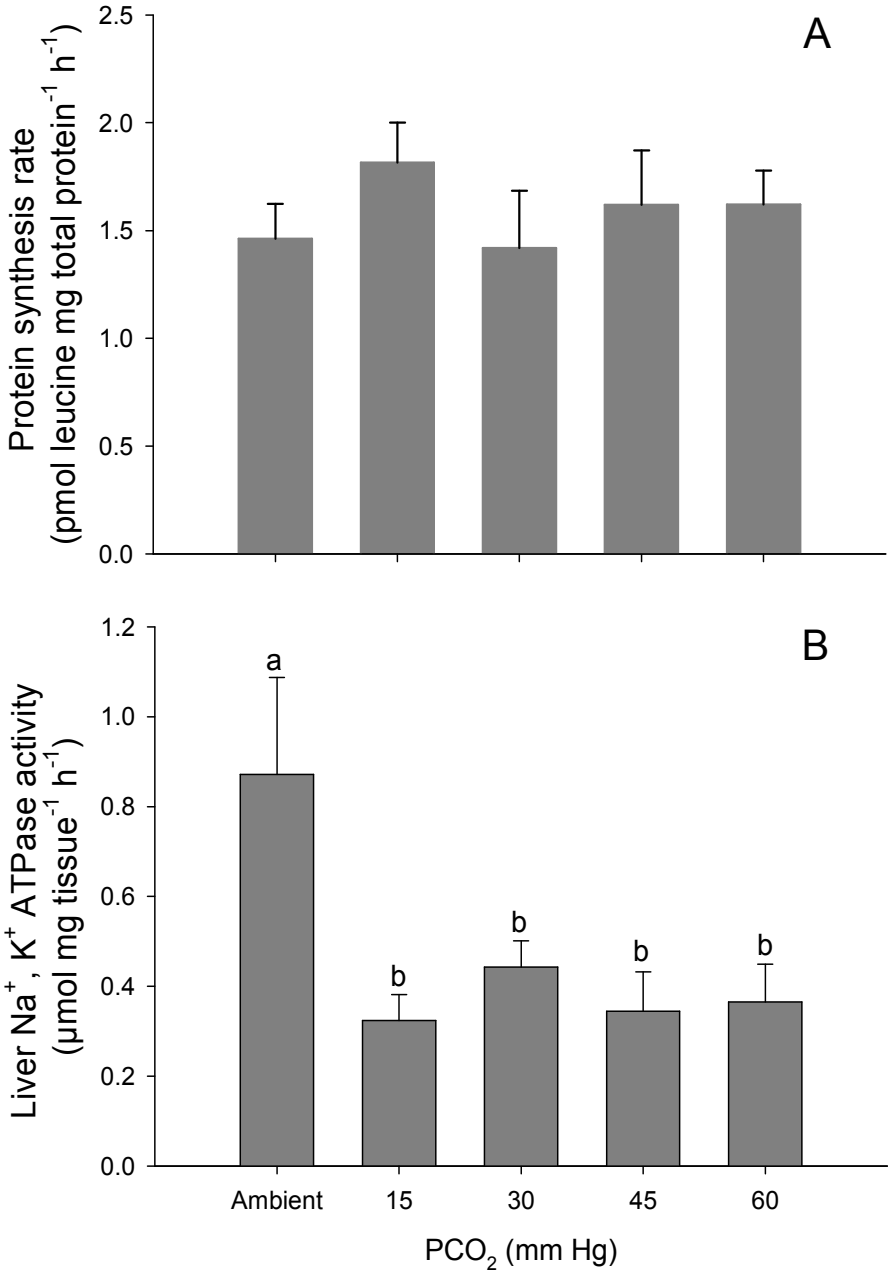


Figure 4.7 The effect of short-term (12 h) hypercarbia (normocarbia, 15, 30, 45, and 60 mm Hg PCO_2) on A) maximal protein synthesis rate and B) maximal Na^+, K^+-ATPase activity of liver homogenates in white sturgeon. Values are means ± s.e.m. (n = 4-8 for each group). Dissimilar letters indicate a significant difference. No statistically significant differences were observed in protein synthesis rates.

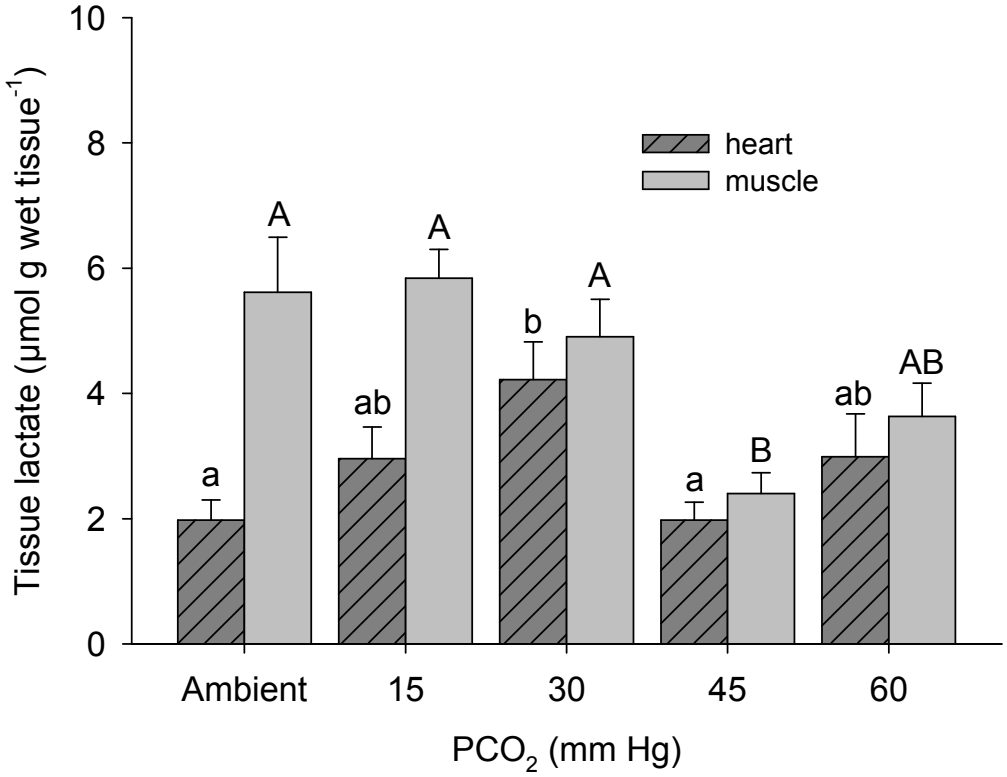


Figure 4.8 The effect of short-term (24 h) hypercarbia (normocarbia, 15, 30, 45, and 60 mm Hg PCO_2) on lactate accumulation in heart (cross hatched bar) and white muscle (grey bars) in white sturgeon. Values are means ± s.e.m. (n = 5-8 for each group). Letters (lower case for heart, upper case for muscle) indicate significant differences between groups within a tissue type.

5. *IN VIVO* INTRACELLULAR pH AND METABOLIC RESPONSES DURING SEVERE SHORT TERM AQUATIC HYPERCARBIA: A ^{31}P NMR STUDY

5.1 SYNOPSIS

Complete pHi protection in white sturgeon was observed as early as 6 h following exposure to severe hypercarbia (Chapter 3; Chapter 4), and in the absence of increases in whole animal metabolic demand (Chapter 4). In this chapter, I used NMR technology to assess *in vivo* pH regulatory and metabolic responses of the heart in real time during the initial 90 min of severe hypercarbia to determine 1) how rapidly preferential pHi regulation could be activated and 2) whether this activation was associated with changes in energetic status. Proton NMR imaging was initially used to localize the heart and design a suppression protocol to eliminate signal from other tissues (e.g., white muscle).White sturgeon hearts exhibited significant increases in pHi during the first 90 min of exposure to aquatic hypercarbia (45 mm Hg PCO_2) and only brief changes in ATP, CrP, and ADP_{free} that were recovered within 90 min, whether measured *in vivo* by ^{31}P NMR or on excised tissues *via* traditional techniques. Heart pHi values calculated from ^{31}P spectra were not different from those measured in tissue homogenates under similar CO_2 exposures (i.e., normocarbia or 90 min hypercarbia). The findings of this study imply that acid-base trans-membrane transporters associated with intracellular pH protection in response to hypercarbia are rapidly (within minutes) activated during exposure to aquatic hypercarbia, and the metabolic consequences of aquatic hypercarbia are compensated quickly within the heart of these exceptionally CO_2-tolerant fish.

5.2 INTRODUCTION

In fishes, aquatic hypercarbia induces an initial intracellular acidosis, the severity of which will depend on both the magnitude of the increase in tissue PCO_2 and the intrinsic buffer capacity of that tissue. As the function of many cellular components (e.g., enzyme activity) is pH sensitive, this acidosis may negatively affect cellular processes, including metabolic pathways. Tissues exhibit a qualitatively similar pattern of acidification and recovery as that observed in the blood in most vertebrates (Rothe and Heisler, 1987; Wood et al., 1990, Wood and LeMoigne, 1991; Figure 1.2). Fish respond to hypercarbia by increasing net acid excretion, but the resulting pHe compensation requires hours and, as the severity of the hypercarbic challenge increases, there is a decline in the efficacy of branchially driven pHe compensation (Heisler, 1986; see Chapter 1). Consequently, the rapid acidosis induced by severe hypercarbia has been thought to be unavoidable in the early stages of CO_2 exposure.

White sturgeon have exceptional short-term CO_2 tolerance (Chapter 3; Baker et al., 2009a; Chapter 4) and can tolerate an elevation of internal PCO_2 and a severe blood acidosis for days (Crocker and Cech, 1998; Chapter 3; Baker et al., 2009a; Chapter 4). Part of this tolerance has been attributed to preferential pHi regulation in tissues such as heart, liver, and brain, (Chapter 3; Baker et al., 2009a). Remarkably, even at CO_2 levels that induce morbidity (i.e., 90 mm Hg PCO_2; Chapter 4), complete pHi compensation is achieved by 6 h. This pHi regulatory capacity of white sturgeon in response to hypercarbia is currently unmatched among fishes studied to date (Chapter 3). However, the early time course (minutes to hours) of pHi regulation during hypercarbia is yet unknown. CO_2 diffuses into tissues rapidly (within 10-15 min, Bernier and Randall, 1998; Chapter 3), and, due to the presence of intracellular carbonic anhydrase, an acidosis would be expected to be induced almost instantaneously. During severe hypercarbia then, pHi could decrease greatly. For example, based on intrinsic buffering capacity (Chapter 3;

Baker et al., 2009b), heart tissue would be predicted to exhibit an acidosis as great as ~0.4 pH units during exposure to 45 mm Hg PCO_2. Intracellular pH perturbations in tissues can have many negative effects in vertebrates, such as decreases in enzyme activity (according to pH optima) (Putnam and Roos, 1997; Hochachka and Somero, 2002), impairment of contractile force (Shiels et al., 2010), and reductions of metabolites associated with cellular energetic status (e.g., [CrP]: Wasser et al., 1990; Jackson et al., 1991; Stecyk et al., 2009; [ATP]: Hillered et al., 1984; Espanol et al., 1992). An acidosis of this magnitude is associated with severe metabolic consequences in other fishes (e.g., decrease cardiac performance, Milligan and Farrell, 1986; reduction of metabolic pathways, Speers-Roesch et al., 2010).Therefore, in the early stages of severe hypercarbia before complete pHi protection is achieved, white sturgeon could potentially exhibit significant metabolic consequences, such as catastrophic declines in heart [ATP] or [CrP].

In Chapters 3 and 4, complete pHi protection during severe hypercarbia was observed as early as following 6 h of exposure. Spectra acquired through nuclear magnetic resonance (NMR) can be used to assess *in vivo* pHi in the heart during initial and early exposure to hypercarbia with temporal resolution not possible through traditional techniques, and without the need for terminal sampling. Through acquisition of spectra from ^{31}P NMR, which relies on characteristics of high energy phosphates, multiple measurements can be obtained sequentially from individuals in real time and resolve differences within short time periods (minutes). ^{31}P NMR can also allow assessment of metabolic changes through calculations of relative changes in high energy phosphates [such as ATP and phosphocreatine (CrP)], and has been used previously for this purpose (e.g., Jackson et al., 1991, Grottum et al., 1998; Bock et al. 2008).

The objective of this study was to characterize changes in heart pHi during early exposure to severe aquatic hypercarbia. To this end, ^{31}P-NMR was used to assess *in vivo* pHi in the heart in real-time throughout the first 90 min of exposure to 45 mm Hg PCO_2. In addition,

changes in high energy phosphates (ATP and CrP) were calculated to determine cellular energetic status, the regulation of which is crucial for proper cardiac function. Finally, I sought to validate *in vivo* ^{31}P-NMR findings through examination of tissue (heart, white muscle, and RBC) pHi and metabolite status following 90 min of hypercarbia using traditional techniques on excised tissues. By examining the early temporal pattern of pHi regulation, and changes in metabolite concentrations in the heart, this study offers insight into the mechanisms and metabolic responses associated with the exceptional tolerance to hypercarbia in white sturgeon.

5.3 METHODS AND MATERIALS

5.3.1 Animals and rearing conditions

White sturgeon, *A. transmontanus*, (4-yr-old; length 60-80 cm; mass 1-2.5 kg) were progeny of wild-caught brood stock from Vancouver Island University (VIU) in Nanaimo, BC, Canada, that were held at the University of British Columbia (UBC), Vancouver, BC, for several months prior to experimentation. All animals were held in large, outdoor flow-through tanks (PwO_2 = 130 mm Hg; atmospheric $PwCO_2$; T = 11–13°C; fish density = 25 kg m^{-3} water) receiving 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}) and fed a commercial diet to satiation daily prior to experiments. No mortality occurred during transport or holding. Food was withheld 24 h prior to experimentation. All experiments were performed at the same temperature as in the holding tanks (12°C) and feeding was withheld 24 h prior to experimentation. All holding and experimental protocols were approved by the UBC Animal Care Committees (animal usage protocol no. A07-0080) and the UBC MRI Research Centre 7T Protocol Committee.

5.3.2 Protocol for hypercarbia exposure

5.3.2.1 Series 1: The effect of hypercarbia on the heart *in vivo* using ^{31}P NMR spectra acquisition

White sturgeon (n = 4) were transferred from the holding facility in the Biological Sciences Building, UBC to the MRI Research Centre in the Life Science Institute, UBC 30 min before placement in the 7T NMR magnet. White sturgeon were mildly anaesthetized, and transferred to a temperature-controlled (12°C), gas tension controlled holding tank designed to be placed within the 7T NMR magnetic bore. A large foam insert was used to keep the trunk of the sturgeon immobilized to ensure scan quality, but ventilation was not impeded by this protocol. Fish were then transferred to the experimental holding tank (≤ 3 min), and fish recovered from anaesthetic rapidly (≤ 2 min for ventilation to commence following transfer). The tank was inserted into the scanning area, and the ^{1}H (Fig. 5.1) and ^{31}P NMR (Fig. 5.2) protocol as described below was begun. The first series of scans (for a representative trace, Fig. 5.2) were collected from fish in water equilibrated with air between 30 and 60 min after transfer was complete, and animals were considered recovered from anaesthetic and handling when ^{31}P NMR scans returned no qualitatively observable variation between collected spectra. After this, re-circulating water was rapidly equilibrated (Fig. 5.3) with a pre-mixed gas mixture (45 mm Hg PCO_2, 150 mm Hg PO_2, balance nitrogen, Praxair, Vancouver, B.C., Canada), and animals were scanned for a further 90 min. White sturgeon can be held at 45 mm Hg PCO_2 for many days without morbidity (Chapter 2; Baker et al., 2009a).

5.3.2.2 Series 2: The effect of hypercarbia on the heart, white muscle and RBC

White sturgeon (n = 6-7) were transferred to a thermostated, aerated, darkened box

supplied with flow through, de-chlorinated city water in the Biological Sciences Building, UBC 48 h before experimental use. Following this, water flow was stopped, and fish were maintained within this system for a further 90 min in either normocarbia (continuous aeration) or hypercarbia (using a pre-mixed gas similar to that used for NMR spectrometry; 45 mm Hg PCO_2, 150 mm Hg PO_2, balance nitrogen, Praxair, Vancouver, B.C., Canada). The water CO_2 equilibration profile for this procedure was similar to that described for NMR experiments. After 90 min, animals were euthanized with anaesthetic (final concentration of MS-222, 0.3 g l^{-1}, with 0.5 g l^{-1} sodium bicarbonate). Upon cessation of ventilation, animals were removed and transferred to a surgery table and fish were killed *via* spinalectomy. Immediately following this procedure, a blood sample was taken with a heparin-rinsed (150 i.u. heparin ml^{-1}) syringe (3 ml, 23G1 needle), and blood was transferred to a 1.7 ml centrifuge tube and centrifuged at 10,000 rpm for 2 min. The resultant plasma supernatant was removed and discarded, and RBC pellet was placed on ice. A portion of white muscle was excised, and the skin and red muscle removed. Then the heart was excised and the conus arteriosus removed. These tissues were either wrapped in pre-labelled aluminum foil and freeze clamped with liquid nitrogen cooled tongs (heart and white muscle), or flash frozen in a bullet tube (RBC), and all tissues were placed in a -80°C freezer for further analysis of pHi and metabolites as described below.

5.3.3 Analytical techniques

5.3.3.1 NMR imaging and spectroscopy

All NMR experiments were carried out on a 7T animal scanner (Bruker, Germany). First a series of ^{1}H NMR images were collected (Fig. 5.1 A,B) using a loop coil located roughly

underneath the heart. This coil was initially tuned and matched to the proton frequency (300.2 MHz) *via* a detachable matching and tuning circuit board. Zero-order shimming of the magnetic field was performed at the proton frequency, as well as during the axial and sagittal localization images [gradient echo FLASH sequence, echo time/repetition time (TE/TR) = 4.3, 500 ms, number of averages = 3, field of view (FOV) = 8 cm, matrix = 256 x 256]. A saturation slice was prescribed based on the localizer proton images to cover as much muscle between the coil and heart as possible, eliminating ^{31}P signal from the white muscle ventral to the heart (Fig. 5.1 C). The thermostated holding container was then carefully withdrawn from the magnet bore and the coil loop was retuned to the phosphorous frequency (121.5 MHz) for metabolite concentration and intracellular pH measurement, by changing over to a new matching/tuning circuit. Finally, the container was gently replaced in the magnet bore in exactly the same position as the first phase of the experiment, to allow the use of the shim and saturation slice geometry acquired previously.

Non-localized ^{31}P NMR spectra were collected in a pulse-acquired fashion (0.25 ms block pulse, TR = 1.5 s), with the saturation slice (5 ms hyperbolic secant pulse) providing some removal of the signal between ventral surface and heart (Fig. 5.1). The power of the saturation slice was iteratively optimized by collecting spectra centered on the phosphocreatine peak, which served as an endogenous reference for power adjustment. The saturation slice power was adjusted so that the CrP peak amplitude was minimized, which was assumed to signify complete suppression of the CrP signal within the suppression slice but allowing the CrP signal above the saturation slice (presumably mostly heart tissue) to remain. Once optimized, the offset frequency of the acquisition was changed to the Pi frequency, to avoid chemical shift of the saturation slice position (Bottomley et al., 1984). Averaged spectra were acquired with enough averages to result in an acquisition time of 2.5 min (Fig. 5.2). Data were collected with Bruker Paravision 4.0 software.

5.3.3.2 Analytical techniques on excised tissues

Red blood cell pHi was determined *via* the freeze-thaw method (Zeidler and Kim, 1977) using a thermostated Radiometer BMS3 Mk2 capillary microelectrode with PHM84 pH meter (Radiometer, Copenhagen, Denmark). White muscle and heart (~150 mg) were ground to a fine powder under liquid nitrogen and pHi was determined in an aliquot following previously described methods (Pörtner et al., 1991; Chapter 2; Baker et al., 2009b) using the same electrode and meter. For metabolite determination, frozen tissue (~30 mg skeletal muscle, ~100 mg liver and ~200 mg red blood cells) was weighed and then sonicated using a micro-sonicator (Kontes, Vineland, NJ, USA) at medium frequency for ~15 sec in 1 ml ice-cold 8% perchloric acid. The resultant homogenates were then centrifuged at 20,000 g for 5 min at 4°C and the supernatant adjusted to ~pH 7.6 by addition of potassium carbonate (3 mol l^{-1}). Neutralized extracts were centrifuged at 20,000 g for 5 min at 4°C and the supernatant was immediately frozen in liquid nitrogen and stored at –80°C until use. These extracts were then used for the enzymatic determination of tissue [ATP] and [CrP] (Bergmeyer, 1983). Total creatine concentration ([Cr]) was determined by heating an aliquot of extract in sealed Eppendorf tubes for 20 min at 60°C and assaying for Cr enzymatically (Bergmeyer, 1983). Free [Cr] was calculated for each sample by subtracting [CrP] from total [Cr]. ATP, CrP and Cr are expressed in μmol g^{-1} tissue.

5.3.4 Calculations

NMR data were analyzed using Java Magnetic Resonance User Interface (jMRUI, v. 2.0). First, each scan was manually adjusted to eliminate signal delay (between 65 and 70 msec) and phase shifted to maximize resolution of metabolic peaks. Owing to the subjectivity of

manual phase shifting, each 2.5 min spectrum was analyzed for frequency separation three times, and the mean of these three values was used for calculation of pHi. This approach was also used on concurrent scans mathematically summed to 15 min intervals, or roughly 5 scans of 2.5 min per sampling period, to increase the signal to noise ratio (SNR).

Calculation of pHi used the frequency separation (δ) between inorganic phosphate (Pi) and phosphocreatine (CrP) (as measured from the spectra collected from each 2.5 min scan and pooled 15 min scans) and a logarithmic formula previously derived experimentally (Hallman et al., 2008 from van den Thillart et al., 1989).

$$pH = 0.372\delta^3 - 4.890\delta^2 + 22.160\delta - 27.798.$$

Heart pHi values from pooled 15 min scans are presented because of the increased signal to noise ratio.

Metabolite concentrations (CrP, ATP and Pi) were calculated from the integral of the summation of 5 ^{31}P NMR spectra using AMARES quantification and peak identification protocols within jMRUI. Because of signal to noise ratios, metabolites were only calculated from 15 min mathematically summed spectra, but each spectrum was analyzed three times as above, and the mean of these three values were taken. CrP values were normalized to the sum of CrP and Pi to reduce the effects of small changes in the area from which ^{31}P signal was collected. Both ATP (evaluated by integration of the area under the α-ATP peak) and normalized CrP are expressed relative to control levels throughout to reduce the visual effect of variability due to NMR acquisition differences.

Free cytosolic [ADP] ([ADP_{free}]) was calculated according to published protocols (Golding et al., 1995; Teague et al., 1996) using the following equation:

$$[ADP_{free}] = [ATP][Cr] / [CrP]\ K'_{CK}$$

The equilibrium constant for creatine kinase (K'_{CK}) was corrected for temperature, pH and free Mg^{2+} (assumed to be 1mmol l^{-1}) as previously described for goldfish (Hallman et al., 2008). Cellular [ATP] and [CrP] were estimated from NMR spectra by the relative resonance intensities of CrP and α-ATP, starting from mean values obtained from excised hearts from normocarbic fish in series 2 (this chapter). [Cr] was calculated by subtracting [CrP] from mean total creatine (data not shown). Metabolite concentrations were expressed in molar concentrations (per liter of tissue water) using a tissue water content of 0.7 ml g^{-1} wet mass, but reported normalized to pre-exposure levels.

5.3.5 Statistical analyses

Data are expressed as means ± sem. Differences in mean values of pHi (in both 2.5 min scans and pooled 15 min scans), CrP, Pi, and ATP (on pooled 15 min scans) following exposure to aquatic hypercarbia using NMR were tested with one-way repeated measures analysis of variance (one-way RM ANOVA). When differences were detected ($p \leq 0.05$), post-hoc tests were used to identify homogenous subsets. Changes in pHi, ATP, CrP, and Cr of heart, RBC and white muscle between ambient CO_2 exposure and 90 min of 45 mm Hg PCO_2 as measured on excised tissue *via* traditional methods were tested using a Student's t-test ($p \leq 0.05$). Differences in pHi as measured from NMR and homogenates during similar treatments were also examined with Student t-tests. Statistical analysis was performed by SigmaStat 10.0.

5.4 RESULTS

White sturgeon exhibited no morbidity during exposure to 45 mm Hg PCO_2, and activity levels and ventilation frequency decreased, consistent with findings presented earlier in this thesis (Chapter 3; Baker et al. 2009b; Chapter 4).

5.4.1 The effect of hypercarbia on tissue pHi

In series 1, aquatic hypercarbia did not result in an acidosis in white sturgeon hearts (Fig. 5.4). The mean pHi of the heart acquired from fish under control conditions was 6.96 ± 0.04, while the mean pHi over the entire 90 min of exposure was 6.98 ± 0.04. There was, however, an effect of time on pHi (one-way RM ANOVA, $p = 0.015$), and pHi measured in first and second 15 min intervals (15 and 30 min) was significantly higher than that of pre-exposed fish. Mean pHi values from 2.5 min acquisition period were more variable, but followed a similar pattern as heart pHi measured in pooled spectra and were never significantly lower than control values during the 90 min exposure to 45 mm Hg PCO_2.

In series 2, aquatic hypercarbia induced a significant acidosis in RBC after a 90-min exposure (Table 5.1). However, in excised tissue, white muscle pHi was unchanged and heart pHi significantly increased compared with control values following 90 min of aquatic hypercarbia (Table 5.1) Furthermore, pHi calculated from ^{31}P NMR spectra and measured on excised tissues did not differ within similar treatments (i.e., between pre-exposed and control fish, or between pHi after 90 min of hypercarbia).

5.4.2 The effect of hypercarbia on tissue metabolites

In series 1, aquatic hypercarbia produced small but significant changes in [ATP], [CrP] and [ADP_{free}] relative to control (Fig 5.5A-C). After 90 min of hypercarbia exposure, no significant differences from control values were observed in these metabolites. In series 2, [ATP], [CrP] and [Cr] measured in RBC, heart and white muscle were unchanged following 90-min of hypercarbia exposure (Table 5.1). Also, [ADP_{free}] calculated for excised heart tissue was not significantly different following 90 min of severe hypercarbia compared to control (ADP_{free}: 16 ± 4 and 23 ± 5 μmol l^{-1} intracellular water, respectively).

5.5 DISCUSSION

White sturgeon hearts did not exhibit an acidosis *in vivo* within the time resolution offered by this protocol (2.5 min intervals), indicating a truly rapid ability for pHi regulation. This finding was surprising, considering that the rapid acidosis induced by severe hypercarbia has been thought to be unavoidable in early stages. Heart [ATP] and [CrP], as calculated from ^{31}P NMR, decreased during hypercarbia, implying that either ATP demand or supply was altered. However, these changes were small and brief, and both had recovered to pre-exposure or control levels by 90 min independent of the measurement method. White sturgeon heart pHi regulation during elevated CO_2, therefore, is one of the most rapid of those observed to date in fishes. While this rapid response may come with a change in metabolic supply or demand, cellular metabolic status was regained within 90 min. The findings of this study imply that the white sturgeon acid-base trans-membrane transporters associated with preferential pHi regulation during hypercarbia are activated within minutes during exposure to severe aquatic

hypercarbia. In addition, alterations in metabolites during aquatic hypercarbia were compensated quickly within the hearts of these exceptionally CO_2-tolerant fish.

5.5.1 Interpretation of ^{31}P NMR spectra

Using ^{31}P NMR to investigate *in vivo* heart pHi in submerged conscious fish has not been done previously. However, attributing calculated changes in pHi and metabolites to specific tissues through *in vivo* examination requires assumptions about the collection area from which ^{31}P spectra are acquired. To support conclusions drawn from NMR, metabolite concentrations in tissues surrounding or perfusing the heart and potentially contributing to the total high energy phosphate signals in the scanned area were measured with the intention of estimating the relative effect of each tissue. Extracellular fluid and plasma lack significant levels of relevant phosphate groups and so can be discounted. RBC contain twice the concentration of ATP as heart tissue (Table 5.1), and white sturgeon hearts may hold up to their own weight in blood (see Chapter 6). Of course, upon contraction this volume would be reduced to 5-10% of the weight of heart tissue, as the ejection fraction of fish hearts is very high (> 95%; Franklin and Davie, 1992). RBC contribution to the ^{31}P signal from perfusion of heart would be less than 2% by volume assuming that blood perfusion in tissues accounts for less than 3% of total tissue volume (Hochachka and Somero, 2002), and that haematocrit in white sturgeon is no greater than 35% throughout the hypercarbic exposure (Chapter 3; Baker et al., 2009a; Chapter 4). Altogether then, the proportion of signal from RBC relative to the heart might be as great as 20%, and thus may represent as much as 40% of the changes in ATP. The contribution of RBC to CrP is estimated at less than 10%, and, because spectra were centered on the peak associated with inorganic phosphates (Pi), the resulting effect of RBC pHi on pHi calculations would be

similarly small. This is important, as changes in RBC pHi are markedly different than those of other tissues (Table 5.1; Chapter 3; Baker et al., 2009a; Chapter 4).

The relative effect of white muscle on ^{31}P NMR spectra of the heart acquired could be significantly greater. When measured on excised tissues, ATP levels in white muscle were 4-fold greater that those in heart tissue, and CrP levels were 7-fold higher. Thus, without the suppression protocol, changes in pHi and metabolites due to the heart tissues could be largely masked by changes in white muscle, as white muscle accounted for close to 80% of the area by volume from which the spectra was acquired. However, with the suppression protocol, contribution by the white muscle by volume was estimated (using proton NMR acquired images) to be reduced by ~90%. In addition, remaining white muscle contributing to the signal would have been well toward the outside regions of signal acquisition (i.e., away from the center of the heart). Signal strength from NMR decreases according to distance from the center of the hemispherical acquisition area at a roughly exponential rate, and so a much stronger signal would have been acquired from the heart. I estimated white muscle signal was reduced by 95% during the application of the signal suppression protocol, and this estimation was supported by CrP values calculated from suppressed and unsuppressed ^{31}P NMR spectra. Consequently, changes in pHi, CrP and ATP calculated from ^{31}P NMR spectra can be interpreted as being mostly from heart tissue, subject to the limitations described above.

5.5.2 The effect of short-term hypercarbia on heart pHi

The transport, transfer, and restraint protocols associated with NMR analysis of white sturgeon likely induced a general stress response in white sturgeon; however sturgeon typically recover quickly (minutes to hours) from stressors such as handling and air exposure (Barton et al., 2000), manual chasing (Baker et al., 2005b), and hypoxia (Baker et al., 2005a). Furthermore,

heart ventricle pHi measured in normocarbia using NMR was not significantly different from that in hearts excised from resting, unstressed animals and using the metabolic inhibitor tissue homogenate method (heart pHi of 6.96 ± 0.04 and 6.91 ± 0.01, respectively). Pre-stress handling may have induced adrenaline release, a stress hormone implicated in CO_2 tolerance of white sturgeon (Crocker and Cech, 1998). However, characteristics of spectra obtained from pre-exposed fishes, and the similar trends described by results from NMR and traditional techniques using tissues collected from terminal experiments imply that stress-related effects associated with handling and anaesthesia were not sufficient to confound the findings of this study.

White sturgeon demonstrated a rapid pHi regulatory response in heart tissue. These fish exhibited no measurable acidosis in tissues examined by ^{31}P NMR even during initial exposure to severe hypercarbia (Fig. 5.4), despite a severe drop in pHe (~0.7 pH units within minutes, see Chapter 3) and RBC pHi (~0.3 pH units, Chapter 2; Chapter 3). In trout hearts, exposure to 8.5 mm Hg PCO_2 *in situ* induces a rapid and severe acidification (~0.3 pH units; Milligan and Farrell, 1986). Based on the intrinsic buffer capacity calculated for white sturgeon heart tissue (Chapter 3; Baker et al., 2009a), an increase in PCO_2 to 45 mm Hg would be predicted to induce an uncompensated acidosis of ~0.4 pH units. Instead, significant increases in heart pHi were observed (Table 1; Fig. 5.4). This rapid regulatory response is contrary to the initial response of most cells to elevated CO_2 (Heisler, 1986). Because of the uncertainty surrounding CO_2 equilibration times within the tissue, net acid extrusion rates cannot be estimated accurately. However, clearly, the capacity of white sturgeon to protect pHi rapidly and completely in critical tissues (i.e., heart) during a general acidosis *in vivo* is confirmed as exceptional compared to other vertebrates (Rothe and Heisler, 1987).

5.5.3 The effect of short-term aquatic hypercarbia on metabolites in the heart

Under normocarbic conditions, values for white sturgeon [ATP], [CrP], [Cr] and [ADP_{free}] in heart were similar to those observed in other fishes (e.g., goldfish, *Carassius auratus*, Jibb and Richards, 2008; tilapia, *Oreochromis* hybrid sp. Speers-Roesch et al., 2010). During aquatic hypercarbia, white sturgeon hearts exhibited alterations in heart metabolite concentrations as measured by ^{31}P NMR. [ATP] and [CrP] (~25 and ~20%, respectively) decreased briefly, but after 90 min of hypercarbia, levels of these metabolites were not significantly different from pre-exposed fish regardless of method of collection (i.e., NMR or assays using excised tissue). CrP can be utilized to synthesize ATP, and, as maintaining ATP homeostasis under adverse conditions appears critical for survival in many fishes (e.g., Jibb and Richards, 2008; Speers-Roesch et al., 2010), increased energetic demands may coincide with a decrease in [CrP]. In other fishes challenged with hypercarbia, hypoxia and exhaustive exercise (Jackson et al., 1991; Jibb and Richards, 2008; Hallman et al., 2008), changes in tissue [CrP] are typically much greater than those in white sturgeon hearts in this study (Jackson et al., 1991; Jibb and Richards, 2008; Hallman et al., 2008).

White sturgeon [ATP] did however, decrease significantly (~25%) during hypercarbia, despite the relatively small changes in heart [CrP]. Based on a 1:1 ratio of ATP production pathways utilizing CrP, only an additional 10-15% decrease in CrP levels would have been necessary to return [ATP] to pre-exposed levels (~0.35 μmol g^{-1}), with no resultant acidosis (unlike ATP produced *via* glycolysis) (Hochachka and Somero, 2002; Hallman et al., 2008). The equilibrium constant of creatine phosphate kinase (CPK), which catalyzes ATP synthesis *via* CrP hydrolysis, is controlled mainly by pHi and ADP, and a tight relationship between pHi and [CrP] in mammalian brain tissue has been observed *via* ^{31}P NMR (Nioka et al., 1987). As heart pHi did not decrease throughout hypercarbia in white sturgeon, CrP recovery rates may be constrained

by ADP. Relative changes in heart [ADP_{free}] increased by approximately 45% following 30 min of hypercarbia, but were unchanged at all other sampling periods, including at 90 min. The consequently lower heart [ATP]/[ADP_{free}] could have shifted the CPK equilibrium towards CrP synthesis. In this way, CrP depletion might be actively avoided, as CrP plays other important roles, such as high energy phosphates cycling. In addition, the inorganic phosphates released during ATP synthesis can have negative effects on contractile machinery; this could explain the absence of further dedication of heart [CrP] to ATP synthesis.

Although the decreases in heart [ATP] and [CrP] are relatively small, that they occur at all signifies that hypercarbia may have metabolic consequences. These consequences could be related to changes in, for example, cardiac function. White sturgeon exposed to 45 mm Hg PCO_2 experience a tachycardia (~20% increase, D. Baker, personal observation) presumably linked to a stress response, and this could affect metabolite status. In addition, the decrease in [ATP] and [CrP] may be related to increased metabolic costs associated with activation of mechanisms of preferential pHi regulation, as many pHi regulatory mechanisms require ATP [e.g., V-ATPases]. In any case, changes in metabolites during severe hypercarbia were not indicative of catastrophic failure to maintain energetic status, and had recovered by 90 min; this conclusion is supported by whole animal oxygen consumption rates, which do not increase at this CO_2 tension (Chapter 4).

5.5.4 The pHi regulatory response of white sturgeon hearts

A pHi regulatory response to elevated CO_2 as rapid as was measured in the heart of white sturgeon has not previously been reported *in vivo*. The typical response of heart pHi in vertebrates during initial stages (< 3 h) of severe hypercarbia is one of a decrease in heart pHi according to intrinsic (i.e., non-bicarbonate) buffer capacity, and this pattern has been observed in rats (e.g., Rothe and Heisler, 1987), turtles (Wasser et al., 1990; Jackson et al., 1991) and fish

(Milligan and Farrell, 1986; Wood et al., 1990; Wood and LeMoigne, 1991). Even in the remarkably CO_2-tolerant western painted turtle, *Chrysemys picta bellii*, perfused hearts exhibited a rapid and severe acidosis during a 15-fold increase in PCO_2 according to intrinsic buffer capacity (Jackson et al., 1991). At the cellular level, the hypoxia tolerant goldfish, *Carassius auratus* has exhibited rapid net acid extrusion at the cellular level: hepatocyte suspensions prepared from, were able to regulate pHi during chemically-induced anoxia to normoxic levels within 20 min, even though a similar challenge induced an acidosis of almost 0.3 pH units in rainbow trout, *Oncorhynchus mykiss* (Krumschnabel et al., 2001). As well, the air-breathing fishes, *Synbranchus marmoratus* (*in vivo*, 6 h, Heisler et al., 1982) and armoured catfish, *Pterygoplichthys pardalis* (*in vivo*, 6 h, Brauner et al., 2004; *in situ*, 20 min, Hanson et al., 2009) have demonstrated a robust capacity for regulating heart pHi. Even so, the pHi regulatory response of white sturgeon hearts *in vivo* during aquatic hypercarbia observed here appears to be exceptional and could provide much insight into the CO_2 tolerance of these fish.

White sturgeon heart pHi regulation appears to be activated more rapidly than in the most tightly pHi regulated mammalian tissue, the brain. For example, in dog brains during ventilation-induced PCO_2 increases of either 2- or 4-fold, pHi decreased significantly (0.13 and 0.27 pH units, respectively) and showed no compensation within ~ 15 min (Nioka et al., 1987). When exposed to hypercarbia that approximately doubled blood PCO_2, rat brain pHi exhibited an acidosis (0.15-0.3 pH units), and pHi remained significant low for between 45 min to 3 h before active pHi regulation compensated for the acidosis (Messeter and Siesjö, 1971; Nishimura et al., 1989; Nattie et al., 2002); a similar pHi depression was observed in newborn lambs (Hope et al., 1988). In mammalian brains, pHi regulation is attributed to a complex response including, for example, blood flow, cellular intrinsic buffering and acid-base relevant exchange at the neural membrane through cellular ion transporters (Nattie et al., 2002). Over the last 4 decades, researchers have localized isoforms belonging to four specific categories of acid-base

transporters in the brain (Chesler, 2003). Clearly, white sturgeon heart pHi regulation is exceptionally rapid, although a great deal of work remains to identify the mechanisms responsible.

5.5.5 Conclusions

Overall, values for pHi and relative changes in metabolites of *in vivo* white sturgeon hearts obtained by ^{31}P NMR were similar to those from excised heart tissue from animals exposed to an identical CO_2 challenge, validating this protocol for assessing heart pHi *in vivo*. White sturgeon heart ventricle exhibited no acidification within the first 90 min of exposure to severe hypercarbia, indicating that intracellular pH regulation is activated rapidly in the heart (within minutes). Whether changes in metabolite concentrations are due to alterations in rates of ATP supply or ATP demand could not be determined here, but clearly, the modest and transient reductions in [CrP] and [ATP] concentrations were not a direct result or associated with an acidosis.

5.6 Tables

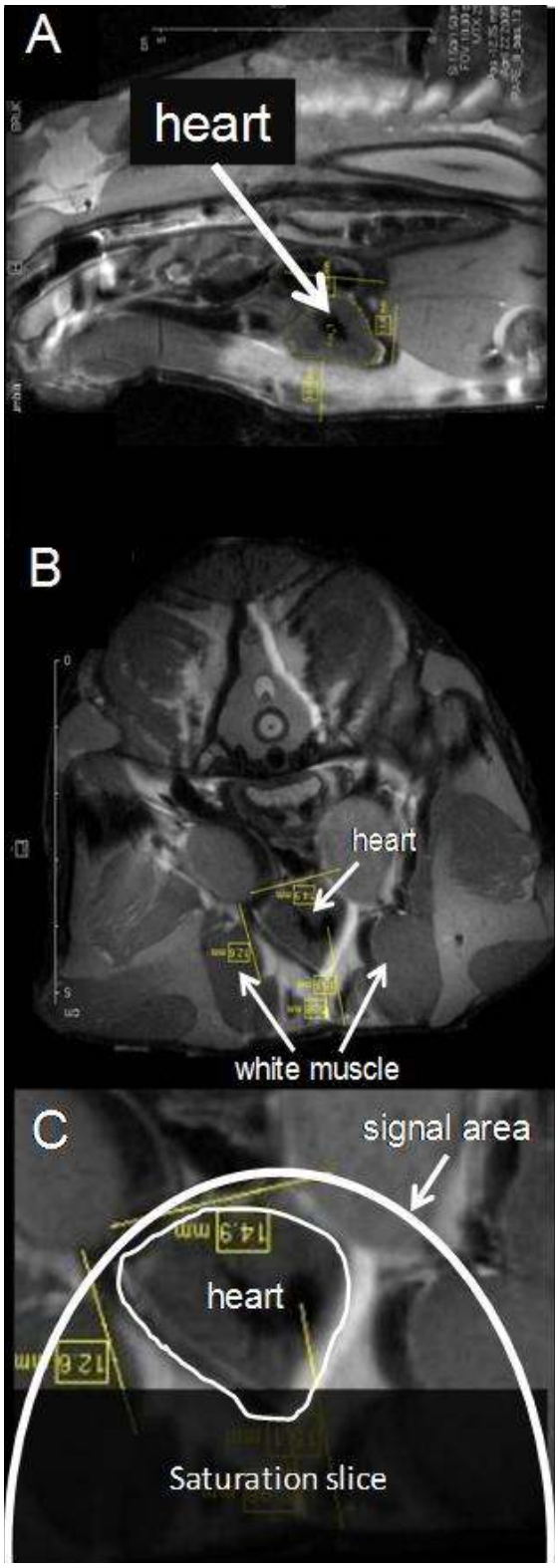
Table 5.1 The effect of short term (90 min) of hypercarbia (45 mm Hg PCO_2) on intracellular pH (pHi), ATP, creatine phosphate (CrP) and free creatine (Cr) in red blood cells (RBC), heart and white muscle of white sturgeon as measured on excised tissues. Values are means ± s.e.m. (N = 5-7 for each group). An asterisk indicates a significant difference from control treatment.

	Intracellular pH		ATP (μmol g^{-1} tissue)		CrP (μmol g^{-1} tissue)		Cr (μmol g^{-1} tissue)	
	Normocarbia	Hypercarbia	Normocarbia	Hypercarbia	Normocarbia	Hypercarbia	Normocarbia	Hypercarbia
Heart	6.91 ± 0.01	6.97 ± 0.01*	1.43 ± 0.20	1.93 ± 0.17	2.63 ± 0.28	2.76 ± 0.29	2.28 ± 0.17	2.58 ± 0.42
White muscle	7.01 ± 0.02	7.06 ± 0.03	6.69 ± 0.18	6.95 ± 0.23	16.6 ± 3.2	12.8 ± 1.3	11.6 ± 1.2	13.1 ± 0.9
Red blood cell	7.22 ± 0.01	6.89 ± 0.01*	2.89 ± 0.22	2.38 ± 0.32	1.17 ± 0.16	1.11 ± 0.17	0.33 ± 0.16	–[1]

1. Below detection limits (0.04 μmol g^{-1} tissue).

5.7 Figures

Figure 5.1 Representative 2 dimensional ^{1}H NMR images collected from white sturgeon. Panel A shows a longitudinal section and illustrates the location of the heart within the fish for precise positioning of the proton and phosphate NMR coils. Panel B is an axial section (vertical slice) centered on the heart of the white sturgeon, and images such as these were used to prescribe the saturation slice and eliminate signal from this area. Panel C shows an estimation of the signal area (the semi-circle outlined with a white line) and the saturation slice (area of removed signal, darkened area within the semi-circle), clearly demonstrating the increase in the proportion of signal coming from the heart (see text for further details).


A
heart
B
heart
white muscle
C
signal area
heart
Saturation slice

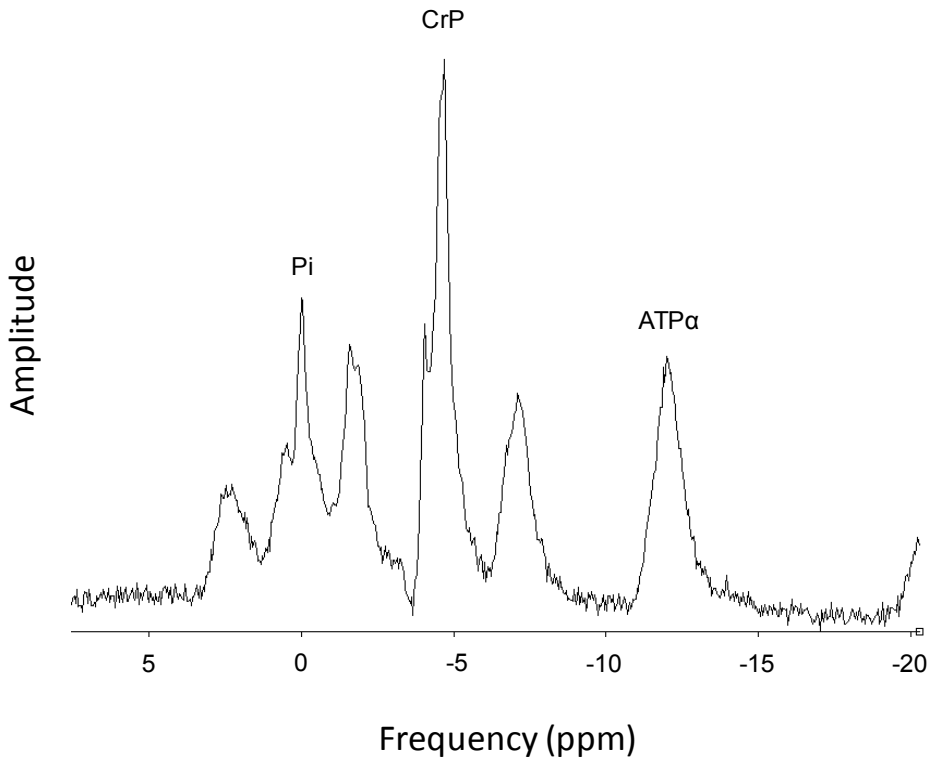


Figure 5.2 Representative ^{31}P-NMR spectra obtained from the signal matched to the ^{1}H NMR image centered on the heart of white sturgeon *in vivo*. ATPα, adenosine triphosphate (as implied by *alpha* phosphate group); CrP, creatine phosphate; Pi, intracellular phosphate. Only peaks quantified for use within the present study are identified, for clarity purposes.

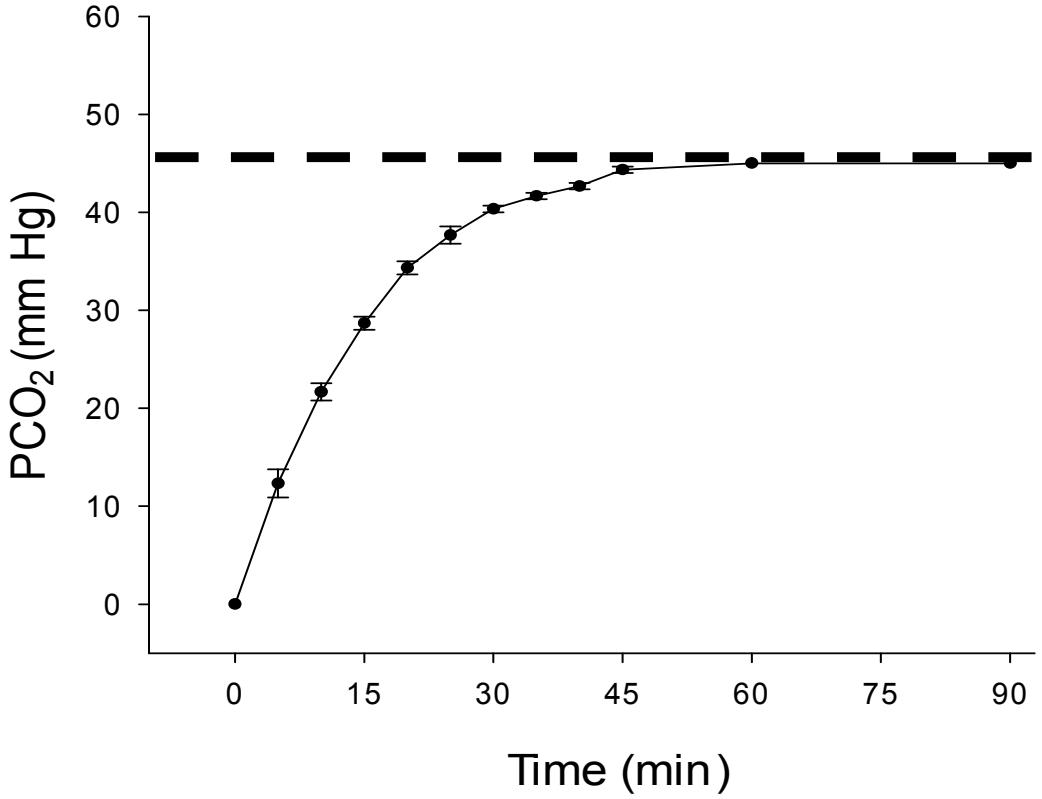


Figure 5.3. Water PCO_2 following initiation of hypercarbia within the chamber used to hold white sturgeon during ^{31}P-NMR spectra acquisition. Data is presented as mean ± s.e.m. (n = 3 for each data point). Dotted line represents PCO_2 of gas mixture used for aeration during aquatic hypercarbia.

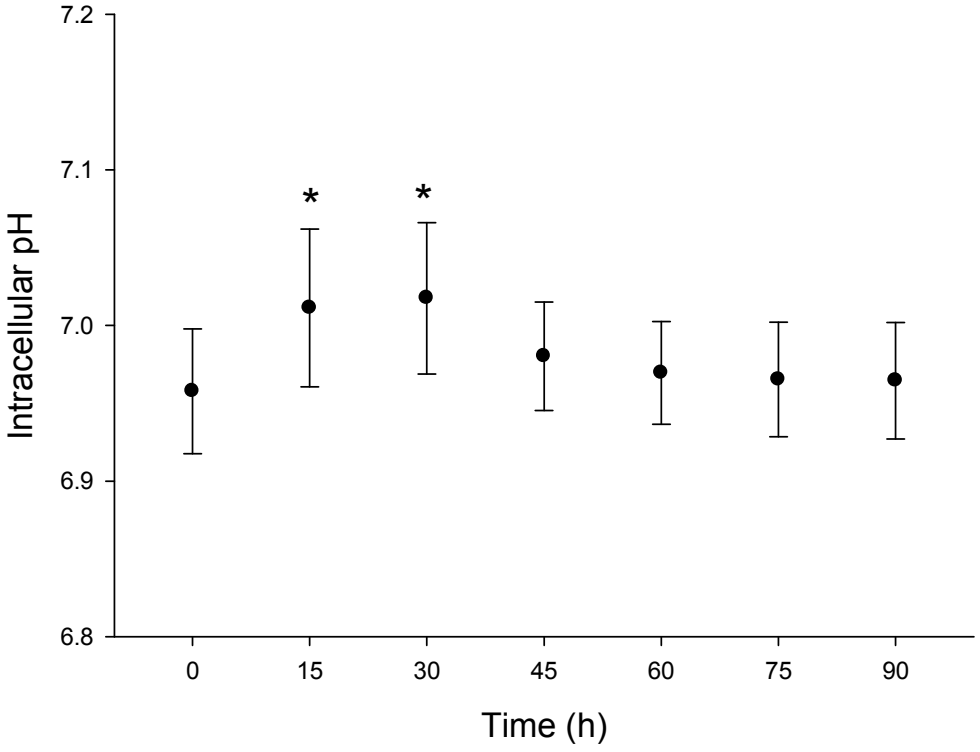


Figure 5.4 The effect of 90 min exposure to severe hypercarbia on intracellular pH of the heart in white sturgeon *in vivo* calculated from ^{31}P NMR. Values are expressed as the absolute change in pHi (ΔpHi) relative to normocarbic controls (time 0 values), and are calculated from pooled values of all 2.5 min scans collected over 15 min. Time zero represents the point at which aeration with pre-mixed gas began. Values are mean ± s.e.m. (n = 4 for each data point). An asterisk indicates significant differences from pre-exposure group (one-way RM ANOVA, p = 0.025).

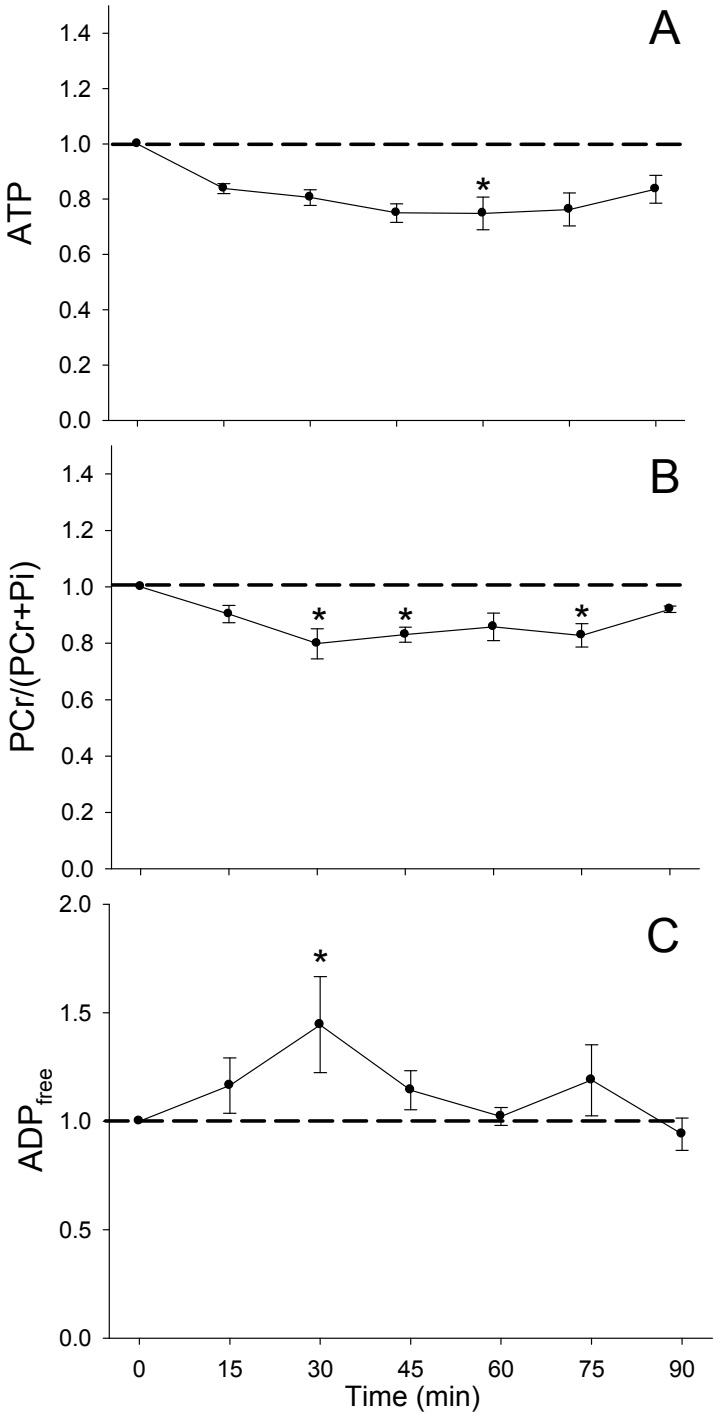


Figure 5.5 The effect of short-term (90 min) hypercarbia (45 mm Hg PCO_2) on relative levels of A) ATP, B) CrP and C) ADP_{free} in white sturgeon hearts *in vivo* calculated from ^{31}P NMR. CrP levels are normalized to the sum of CrP and Pi and ATP, CrP and ADP_{free} are expressed relative to pre-exposure (i.e., time 0) values, the point at which aeration was switched to 45 mm Hg PCO_2 gas. Values are mean ± s.e.m. (n = 4 for each data point). An asterisk indicates a significant difference from pre-exposure values.

6: EXCEPTIONAL PROTECTION OF MAXIMUM CARDIAC PERFORMANCE DURING HYPERCAPNIA IS FURTHER ENHANCED BY ADRENERGIC STIMULATION IN PERFUSED HEARTS

6.1 SYNOPSIS

White sturgeon preferentially regulate pHi during hypercarbia, but the previous chapters shows little evidence of large metabolic costs (Chapter 4; Chapter 5), and rapid pHi regulation in the heart (within minutes) . In this chapter, I used an *in situ* heart preparation to investigate whether the enhanced CO_2 tolerance of white sturgeon is associated instead with a cost to organ performance (e.g., reduction in cardiac scope). Maximum cardiac output (Q_{max}) and maximum cardiac power output (PO_{max}) was assessed using a working, perfused in situ heart preparation. Although fish hearts are generally regarded as being acidosis intolerant, exposure to 22.5 mm Hg PCO_2 for 20 min had no significant effect on maximum cardiac pumping and power capacity of white sturgeon. Exposure to 45 mm Hg PCO_2 significantly reduced heart rate, Q_{max}, PO_{max} and rate of ventricular force generation (F_V) by 23%, 28%, 26%, and 18%, respectively, but these modest impairments accompanied only partial compensation of the intracellular ventricular acidosis, in contrast to the complete compensation observed *in vivo* previously. Even so, full recovery was possible under a return to control conditions. Furthermore, maximum adrenergic stimulation (500 nmol l^{-1} adrenaline) during exposure to 45 mm Hg PCO_2 protected maximum cardiac performance via increased inotropy (force of contraction) without affecting heart rate. Q_{max} and PO_{max} during exposure to CO_2 levels that induce morbidity *in vivo* (60 mm Hg PCO_2) was not quantitatively different from that seen at 45 mm Hg PCO_2, but was qualitatively different as hearts exhibited arrhythmia and a reduction in stroke volume during power assessment. Maximum cardiac performance was unusually CO_2- and acidosis-tolerant, implying

that pHi compensation does not incur a large cost to cardiac work, although the underlying mechanisms associated with this aspects of sturgeon cardiac function remain to be elucidated.

6.2 INTRODUCTION

In the previous chapters, white sturgeon exhibited preferential pHi regulation in many tissues during severe hypercarbia (Chapter 3; Baker et al., 2009b), and complete pHi protection was accomplished rapidly (within minutes; Chapter 5) within the heart with little perturbation of metabolites (Chapter 5) or increase in rates of whole animal oxygen consumption (Chapter 4). However, while preferential pHi regulation appears to be accomplished in the absence of a metabolic cost (Chapter 4; Chapter 5), other consequences of this pHi compensatory strategy may exist. Certainly, hypercarbia has adverse effects on fishes (e.g., Graham et al., 1990; Wood et al., 1990; Wood and LeMoigne, 1991; Hayashi et al., 2004), and the fish heart is particularly sensitive. Routine heart rate (f_H) of rainbow trout, *Oncorhynchus mykiss*, and cardiac output of Atlantic salmon, *Salmo salar*, and dogfish, *Squalus acanthus*, decreased rapidly during exposure to moderate or severe hypercarbia respectively (28-35 mm Hg PCO_2) (Perry et al., 1999; Peirce, 1978; Perry and McKendry, 2001). Similar conclusions were reached when maximum cardiac performance was assessed with working, perfused, fish heart preparations. For example, using perfused sea raven (*Hemitripterus americanus*) hearts, just 11.5 mm Hg PCO_2 significantly decreased maximum cardiac output (Q_{max}) and maximum cardiac power output (PO_{max}) as well as f_H (Farrell et al., 1984). Also, rainbow trout, (Farrell et al., 1986) and ocean pout, *Macrozoarces americanus* (Farrell et al., 1983) perfused hearts exhibited reductions in Q_{max} (29% and 18%, respectively) and PO_{max} (29% and 22%, respectively) during equilibration with only 15 mm Hg PCO_2. Larger increases in PCO_2 (75 mm Hg) decreased (~20-60%) maximum isometric force of ventricular strip preparations from rainbow trout (Gesser et al., 1982),

confirming the general sensitivity of f_H and inotropy to CO_2-induced acidosis.

Nevertheless, not all fish hearts are so sensitive to hypercapnia. CO_2-tolerant fish species include the armoured catfish, *Pterygoplichthys pardalis*, and European eel, *Anguilla anguilla*, both of which can tolerate hypercarbia (~ 37.5 mm Hg PCO_2) for days (Brauner et al., 2004; McKenzie et al., 2003). Correspondingly, ventricular strips from the eel remarkably recover contractility after 20 min while still hypercapnic (75 mm Hg PCO_2; Gesser et al., 1982). The armoured catfish heart also stands out for its high CO_2-tolerance since a 49 mm Hg PCO_2 increase above control levels was required to decrease maximum cardiac power output and maximum cardiac output by ~50% (Hanson et al., 2009). White sturgeon hearts may also be CO_2-tolerant as routine cardiac output was unchanged *in vivo* during short term hypercarbia (22.5 mm Hg PCO_2; Crocker et al., 2000). White sturgeon also regulate heart pHi rapidly during hypercarbia *in vivo* (Chapter 3; Baker et al., 2009a; Chapter 5), but whether protection of *in vivo* resting cardiac function during hypercapnia extends to maximum cardiac performance, or is dependent upon pHi regulation or adrenergic stimulation remains unknown. Also unclear are the potential roles of both intracellular pH and circulating catacholamines in protecting cardiac performance during elevated CO_2. For example, some CO_2-sensitive hearts show adrenergic cardiac protection (Farrell, 1985; Gesser et al., 1982), but not ventricular pHi regulation (Farrell and Milligan, 1986). In contrast, armoured catfish hearts at a higher PCO_2 exhibited reduced cardiac performance *in situ* despite complete ventricular pHi compensation, suggesting less cardiac protection through adrenergic pathways in CO_2-tolerant fishes (Hanson et al., 2009).

The objective of this study was to determine whether hypercapnia and preferential pHi regulation in white sturgeon was associated with a decline in cardiac function, and to this end I assessed maximum cardiac performance in perfused white sturgeon hearts, a preparation free of potentially confounding effects, such as vagal and endocrine influences. Specifically, the aims of this study were: 1) to quantify changes in maximum cardiac performance at CO_2 tensions

approaching the limit of white sturgeon CO_2 tolerance *in vivo* (≤ 60 mm Hg PCO_2); 2) to determine whether cardiac recovery occurs following a decrease in maximum cardiac performance associated with short term exposure to hypercapnia; 3) to determine if high levels of exogenous adrenaline protects maximum cardiac performance during hypercapnia; and 4) to identify whether ventricular pHi of perfused hearts is protected during hypercapnia, as has been previously observed *in vivo*.

6.3 METHODS AND MATERIALS

6.3.1 Animals and rearing conditions

Juvenile (1 and 2 yr olds) hatchery-reared white sturgeon, *Acipenser transmontanus*, were provided by the Upper Columbia White Sturgeon Recovery Initiative's white sturgeon hatchery in Wardner, B.C. They were transported by tanker truck to the University of British Columbia, Vancouver, B.C., Canada, and maintained in holding tanks supplied with de-chlorinated flow through city water (in mmol l^{-1}: Na^+, 0.06; Cl^-, 0.05; Ca^{2+}, 0.03; Mg^{2+}, 0.007; K^+, 0.004; alkalinity, 3.3 mg as $CaCO_3$ l^{-1}; hardness 3.55 mg as $CaCO_3$ l^{-1}; [Metro Vancouver 2007], temperature=7-11°C, pH ~6.7-6.9) under a natural photoperiod at densities no greater than 15 kg m^{-3}. Fish were fed to satiation three times per week with a Moore-Clark trout chow, but food was withheld 24 h before experimental use.

6.3.2 Surgical procedures

The *in situ* heart preparation used in this study has been described previously in detail for different species with a variety of minor modifications (Farrell et al., 1983; Farrell and Milligan,

1986; Hanson et al., 2006; Hanson et al., 2009). In brief, white sturgeon (300-1300 g; relative ventricular weight 0.096 ± 0.003%) were anaesthetized in buffered tricaine methane sulfonate (MS-222, 0.3 g l^{-1}, $NaHCO_3$ 3.0 g l^{-1}), then weighed, and transferred to an operating table. A solution of heparinized saline (1 mg kg^{-1}, 150 i.u. ml^{-1} heparin) was injected into the caudal vessel, and the spinal cord severed and brain destroyed, eliminating vagal input to the heart. 2-3 min later, a shallow lengthwise incision was made along the ventral surface of the abdominal cavity from the anal opening to the pectoral girdle. The abdominal wall was then excised to expose the liver, which varied in size, location and appearance. It was typically flat, thin and delicate, wrapping around other organs and having connective adhesions with many tissues, especially the gastrointestinal tract. The right hepatic vein (consistently the largest) was used for cannula insertion and all other major hepatic veins were tied off, including the vessels along the gastrointestinal tract. A small incision was made in the right hepatic vein, and a bevelled stainless steel input cannula was inserted into the vein (and advanced into the sinus venosus) and secured with silk suture. The heart was immediately (and continuously) perfused with saline (composition below) containing 10 i.u. ml^{-1} sodium heparin and a tonic level of adrenaline (5-10 nmol l^{-1} adrenaline bitratate salt; AD). Subsequently, the gills were removed, and a stainless steel output cannula was inserted into the bulbus arteriosus [which in sturgeon is distal to a conus arteriosus (Guerrero et al., 2004; Icardo et al., 2004)] *via* the ventral aorta and secured with silk suture. These surgical procedures were completed within 10-15 min. The fish was transected (approximately mid-abdomen) to allow for easier handling and the large venous sinus that was severed as a result of transection was sutured to the trunk. Following surgery, fish were transferred to a temperature-controlled saline bath (0.7% NaCl), the input cannula was connected to an adjustable, constant pressure reservoir and the output cannula was connected to a separate constant pressure head set at 2.0 kPa to simulate resting *in vivo* ventral aortic diastolic blood pressure. The input pressure head was in turn connected to a set of isolated water-jacketed glass

reservoirs containing aerated perfusate. All experiments were conducted at 10°C.

Input (P_{in}) and output (P_{out}) pressure were measured through saline-filled side arms (PE50 tubing) connected to disposable pressure transducers (DPT 6100; Smiths Medical, Kirchseon, Germany), and cardiac outflow was measured through the output line with a previously calibrated, in-line electromagnetic flow probe (SWF-4; Zepada Instruments, Seattle, WA, USA). The height of the input pressure reservoir was adjusted to set routine cardiac output (Q) at approximately 17 ml min^{-1} kg^{-1}, which was derived from *in vivo* cardiac output estimates for white sturgeon (Crocker et al., 2000), and adjusted for differences in ambient temperature using a Q_{10} value of 2 (Lillywhite et al., 1999). Mean P_{in} during routine cardiac output ranged from 0.04 to 0.16 kPa. Heart rate was independent of filling and output pressures, as has been observed in isolated perfused ventricles of *A. naccarii* (Agnisola et al., 1999). While sturgeon do have a coronary circulation (Icardo et al., 2004), the coronary arteries were not perfused in this preparation, which can affect ventricular contractility of some fishes (Davie et al., 1992; Farrell, 1987). Following surgery, hearts were allowed to recover at routine workloads for 20 min at control (i.e., normocapnic) CO_2 tension (3.75 mm Hg PCO_2) prior to the first maximum cardiac performance test.

6.3.3 Perfusate composition

For all experiments, a freshwater fish perfusate (in mmol l^{-1}: NaCl, 125; KCl, 3.0; $MgSO_4$ $7H_2O$, 1.0; $CaCl_2$ $2H_2O$, 2.5; D-Glucose, 5.6; $NaHCO_3$, 11.9; all chemicals from Sigma-Aldrich, Oakville, Ont., Canada) was used. Depending on the experimental protocol (see below), the perfusate was gassed with 3.75 mm Hg (control), 22.5 mm Hg, 45 mm Hg or 60 mm Hg PCO_2 prepared gas mixtures (Praxair, Vancouver, B.C., Canada; certified to be within 0.75 mm Hg PCO_2, but reported nominally as 3.75, 22.5, 45, and 60 mmHg throughout), containing 150

mm Hg PO_2 with the balance N_2. When aerated with the control CO_2 mixture (3.75 mm Hg PCO_2), the equilibrated perfusate had a pH of 7.80. As CO_2 tension in the perfusate was increased progressively to 22.5, 45, and 60 mm Hg PCO_2, perfusate pH decreased to 7.25, 6.85 and 6.70 respectively. These perfusate pH values corresponded closely to blood pH values measured *in vivo* during exposure to similar water CO_2 tensions (Baker et al., 2009a; Chapter 4). As routine heart rate in sturgeon is under modest β-adrenergic tonus (McKenzie et al., 1995), all perfusates were supplemented with a tonic level of adrenaline (see experimental protocols below for concentrations); preliminary investigation supported the need for adrenaline to ensure routine cardiac function.

6.3.4 Experimental protocols

Maximum cardiac performance was initially assessed under control CO_2 conditions (3.75 mm Hg PCO_2) and then during each treatment condition, as described below for each series of experiments. By initially measuring maximum cardiac output (Q_{max}) and maximum cardiac power output (PO_{max}), each heart acted as its own control. To assess maximum cardiac output, P_{in} was raised in a stepwise manner (~0.05 kPa steps) over 3-5 min until Q reached a plateau; this was recorded as Q_{max}. Similarly, with input pressure remaining at its maximum, P_{out} was raised incrementally until cardiac PO_{max} was reached. Following the maximum performance tests, P_{out} and then P_{in} were returned to routine levels and the heart was allowed to recover for ~5 min before being subjected to the next experimental saline. Preliminary investigation showed that under normocapnic conditions, maximum cardiac performance could be repeatedly assessed at least four times with no loss of performance (with a 15-20 minute rest period between each test), and that no change in maximum cardiac performance occurred over a 3 h period, which was 1 h longer than any experimental protocol used in this study. For each hypercapnic

condition, hearts were allowed to equilibrate for 10-20 min at routine workloads before their maximum cardiac performance was assessed.

6.3.4.1 Series 1: The effect of hypercapnia (22.5, 45, and 60 mm Hg PCO_2) on maximum cardiac performance

In this series, I determined the CO_2 tension at which Q_{max} and PO_{max} became impaired in white sturgeon (body mass 374 ± 14 g; ventricular mass 366 ± 18 mg). Maximum cardiac performance was assessed under control conditions and then at one of the following CO_2 tensions: 22.5 mm Hg (n = 4), 45 mm Hg (n = 4), or 60 mm Hg (n = 4) after a 20-min equilibration period. To reduce fish usage, three hearts were assessed under two CO_2 tensions, first at 22.5 mm Hg PCO_2 and then at either 45 (n = 1) or 60 (n = 2) mm Hg PCO_2. No significant differences (Student t-test) in performance were seen between hearts exposed to 22.5 mm Hg PCO_2 prior to a higher level of hypercapnia relative to hearts exposed directly to a higher level of hypercapnia. Consequently, data from hearts conducted at a given CO_2 were pooled for all analyses. Multi-step protocols have been used to assess maximum cardiac performance in other CO_2-tolerant fishes (Hanson et al., 2009). All perfusates contained 10 nmol l^{-1} [AD].

6.3.4.2 Series 2: The effect of hypercapnia (45 mm Hg PCO_2) on subsequent recovery of maximum cardiac performance

In this series, I determined whether the sturgeon heart recovered its loss of cardiac performance during severe 45 mm Hg PCO_2 when the heart was returned to control CO_2 conditions in 6 fish (body mass 382 ± 15 g; ventricular mass 364 ± 42 mg). Maximum cardiac performance was assessed using 20-min equilibration periods for the following sequence of CO_2

tensions: a) control CO_2 tension (3.75 mm Hg PCO_2), b) hypercapnia (45 mm Hg PCO_2), and then c) post-hypercapnic recovery (3.75 mm Hg PCO_2). All perfusates contained 10 nmol l^{-1} [AD].

6.3.4.3 Series 3: The effect of hypercapnia (45 mm Hg PCO_2) on maximum cardiac performance with maximal exogenous stimulation by adrenaline (500 nmol l^{-1} [AD])

In this series, I determined if maximum adrenergic stimulation could protect Q_{max} and PO_{max} during severe hypercapnia in 8 fish (body mass 991 ± 82 g; ventricular mass 943 ± 88 mg). Each heart was exposed to the following sequence of perfusates: a) control CO_2 tension (3.75 mm Hg PCO_2) with 5 nmol l^{-1} [AD], b) hypercapnia (45 mm Hg PCO_2) with 5 nmol l^{-1} [AD], and c) hypercapnia (45 mm Hg PCO_2) with 500 nmol l^{-1} [AD]. This level of adrenaline was selected to allow for comparison with other studies (e.g. Hanson et al., 2009), and similar levels have been measured in vivo (Burggren and Randall, 1978). Prior to the addition of AD (500 nmol l^{-1}), perfusates used in this series of experiments contained 5 nmol l^{-1} [AD] (as opposed to 10 nmol l^{-1} as in Series 1 and 2) to reduce the possibility of prematurely saturating adrenergic receptors, as little is known about the adrenergic sensitivity of sturgeon.

6.3.5 Tissue pHi determination

The ventricle was rapidly excised and weighed after heart were exposed to control CO_2 levels (3.75 mm Hg PCO_2, n = 2, CO_2-unexposed hearts, n = 8), hypercapnia (Series 1; 45 mm Hg PCO_2, n = 4) or hypercapnia with saturating levels of adrenaline (Series 3; 45 mm Hg PCO_2 with the addition of 500 nmol l^{-1} [AD], n = 8), then flash frozen in liquid nitrogen for later analysis of pHi. In addition, as a limited number of ventricles (n = 2) were sampled during

exposure to control CO_2 tension (3.75 mm Hg PCO_2), pHi was also measured in ventricles from hearts excised from resting normocarbic fish (as listed above) (n = 8). Ventricular pHi was measured using the metabolic inhibitor tissue homogenate method (Pörtner et al., 1990), which has previously been validated for use in tissues exposed to large changes in CO_2 tensions (Chapter 2; Baker et al., 2009a). In brief, freeze-clamped ventricles were ground under liquid nitrogen and added to a pre-cooled centrifuge tube (2.0 ml) with a pre-cooled scoop. A 1 ml aliquot of a metabolic inhibiting solution (150 mmol l^{-1} potassium fluoride and 5 mmol l^{-1} nitrilotriacetic acid disodium salt; Sigma Aldrich, Oakville, Ont., Canada) was then added, and the mixture was placed on ice. The resultant supernatant pH was measured via a thermostated capillary electrode (Radiometer, BMS 2, London, Ont., Canada) attached to a pH meter (Radiometer, PMS 83, London, Ont., Canada).

6.3.6 Calculations and statistical analyses

All cardiac measurements were recorded in real-time using data acquisition software (Labview version 5.1, National Instruments, Austin, TX, USA). P_{in}, P_{out}, f_H, cardiac output (Q) and cardiac power output (PO) were recorded simultaneously at a sampling rate of 10 s^{-1}. Rate of ventricular force generation (F_V) was calculated from raw data as the average maximum change in P_{out} ($\Delta p/\Delta t$, in kPa s^{-1}) when the heart was performing at PO_{max}. In Series 1, statistically significant differences were determined by paired t-tests and data reported as relative changes, while in Series 2 and 3 differences were determined by one-way repeated measures analysis of variance (one-way RM ANOVA). Comparisons of ventricular pHi were made using one-way ANOVA. Where differences were indicated by ANOVA, a SNK post-hoc test was used to determine homogenous subsets. For comparisons, $\alpha = 0.05$ was determined to be appropriate for detecting statistical differences. All values are reported as mean ± s.e.m., unless otherwise

indicated.

6.4 RESULTS

6.4.1 Series 1: The effect of hypercapnia (22.5, 45, and 60 mm Hg PCO_2) on maximum cardiac performance

Relative to control conditions (3.75 mm Hg PCO_2), Q_{max} and PO_{max} were unaffected by 22.5 mm Hg PCO_2, but significantly reduced by severe hypercapnia (both 45 and 60 mm Hg PCO_2; Fig. 6.1C,D). Hypercapnia significantly slowed f_H during Q_{max} measurements (by 10%, 25%, and 25% at 22.5, 45, and 60 mm Hg PCO_2, respectively; Fig. 6.1A) and produced arrhythmia at 60 mm Hg PCO_2 (Fig. 6.2), but had no significant effect on V_S during Q_{max} at any CO_2 tension (Fig. 6.1B). In contrast, at 60 mm Hg PCO_2, V_S during PO_{max} measurements was significantly reduced (53 ± 3%). When assessed at PO_{max}, F_V was reduced with 45 and 60 mm Hg PCO_2, but not with 22.5 mm Hg PCO_2 (Table 6.1).

6.4.2 Series 2: The effect of hypercapnia (45 mm Hg PCO_2) on subsequent recovery of maximum cardiac performance

As in Series 1, 45 mm Hg PCO_2 significantly decreased Q_{max}, PO_{max} and f_H, but did not affect V_S (Fig. 6.3). Control performance was completely restored following a 20-min recovery with 3.75 mm Hg PCO_2 (Fig. 6.3).

Attempts to similarly recover hearts from 60 mm Hg PCO_2 with 3.75 mm Hg PCO_2 (n = 4, data not shown) were abandoned because this level of severe hypercapnia induced arrhythmia (Fig. 6.2) and, in some hearts, cessation of cardiac rhythm entirely. As a result, two hearts were unable to maintain V_S when P_{out} was increased. The two hearts that continued to work during

exposure to 60 mm Hg PCO_2 did not recover maximum cardiac performance upon return to control conditions, suggesting permanent cardiac damage.

6.4.3 Series 3: The effect of hypercapnia (45 mm Hg PCO_2) on maximum cardiac performance with maximal exogenous stimulation by adrenaline (500 nmol l^{-1} [AD])

Hearts exposed to 45 mm Hg PCO_2 exhibited significant decreases in Q_{max}, PO_{max}, F_v, and f_H, but not V_S (Table 6.1, Fig. 6.4) as in Series 1 and 2. Addition of 500 nmol l^{-1} [AD] during severe hypercapnia fully restored Q_{max} and PO_{max}, protecting F_V and enhancing V_S, but without recovering f_H (Fig. 6.4; Table 6.1). Therefore, maximal adrenergic stimulation prevented the negative inotropic effect but not the negative chronotropic effect of severe hypercapnia,

6.4.4 Tissue pHi determination

Given the negative effects of severe hypercapnia and the protective effect of adrenaline during severe hypercapnia, it was anticipated that an intracellular ventricular acidosis would be ameliorated by maximum adrenergic stimulation. Compared with control hearts sampled either *in situ* or *in vivo* (Table 6.2), mean ventricular pHi was significantly reduced by exposure to 45 mm Hg PCO_2. Mean ventricular pHi in the presence of 500 nmol l^{-1} [AD] at 45 mm Hg PCO_2 was significantly higher than at 45 mm Hg PCO_2 without maximum adrenergic stimulation. Even so, ventricular pHi still remained significantly lower than ventricular pHi measured under control conditions both *in situ* and *in vivo* (Table 6.2).

6.5 DISCUSSION

The exceptional CO_2 tolerance of white sturgeon exposed to hypercarbic water clearly extends to perfused cardiac tissue working at maximal rates of performance. *In situ* perfused white sturgeon hearts maintained maximum cardiac performance during exposure to 22.5 mm Hg PCO_2 (Fig. 6.1A), which is a level of hypercapnia known to impair performance of less CO_2-tolerant fish. Exposure to severe hypercapnia (45 mm Hg PCO_2) did impair Q_{max} (~25%) and PO_{max} (~25%) of working hearts through changes in f_H. These reductions were associated with an intracellular ventricular acidosis, and a reduction in F_V, yet still represent extremely modest cardiac impairment compared to other fishes (Farrell et al., 1983; Farrell et al., 1986). Furthermore, the decrease in maximum cardiac performance at 45 mm Hg PCO_2 was not permanent (control performance was fully restored with normocapnia) and it could be fully reversed by addition of exogenous adrenaline to provide maximum adrenergic stimulation. In contrast, increasing PCO_2 to 60 mm Hg, although inducing reductions in Q_{max} and PO_{max} similar to those at 45 mm Hg, caused arrhythmia to develop. Finally, following return to control CO_2 tension, maximum cardiac performance was not restored, implying that cardiac damage, or at the very least temporary myocardial dysfunction (e.g., Hanson et al., 2006), occurs at 60 mm Hg CO_2, which can induce morbidity *in vivo* (Chapter 4).

6.5.1 Maximum cardiac performance during hypercapnia

Maximum cardiac performance of white sturgeon heart has not been previously assessed *in situ*, but would be expected, based on their sedentary life history to be lower than in a pelagic predator like rainbow trout. Certainly, compared to that of rainbow trout hearts, the intrinsic f_H of white sturgeon hearts is much lower (~50% at 10°C; Arthur et al., 1992; Hanson et al., 2006).

Also, Q_{max} measured here for white sturgeon hearts was 20-40% less than that of the rainbow trout (Hanson et al., 2006) and PO_{max} only 25% of that in rainbow trout hearts (Hanson et al., 2006). White sturgeon lack the aerobic scope of pelagic fishes such as salmonids (Peake, 2004), and are often described as a benthic cruiser. The lower power output of white sturgeon hearts (~1.2-1.5 mW g ventricle^{-1}) may reflect this limited athletic prowess.

Hypercapnia-induced reductions in cardiac performance are typically due to both negative chronotropic (frequency of contraction) and inotropic (force of contraction) effects on fish hearts. Hearts of rainbow trout (Farrell et al., 1986), sea raven (Farrell et al., 1983) and ocean pout (Farrell et al., 1983) all exhibited a reduction in both f_H (10 - 15%) and V_S (5 - 10%) during exposure to 11-15 mm Hg PCO_2. Perfused hearts of the CO_2-tolerant armoured catfish, *P. pardalis*, exhibited no change in f_H or V_S at 19 mm Hg CO_2, but f_H (~30%) and V_S (~35%) decreased significantly at 55 mm Hg PCO_2 (Hanson et al., 2009). As with catfish hearts, sturgeon hearts exhibited no decrease in V_S (+12% above control; $p = 0.058$) or F_V (+11% above control; $p = 0.143$) at 22.5 mm Hg PCO_2, although f_H was significantly lower (8% below control). Furthermore, V_S of white sturgeon hearts was unchanged with 45 and 60 mm Hg PCO_2 even though F_V decreased slightly (~18%), and so decreased Q_{max} and PO_{max} (~25% each) reflected negative chronotropic effects (f_H decreasing ~25% with 45 mm Hg PCO_2). Thus, the remarkable CO_2 tolerance of white sturgeon hearts is associated with a protection of inotropy more so than chronotropy.

In vertebrates hearts, high CO_2 can induce chronotropic effects by, for example, alterations in the activity of hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels in pacemaker cells, which set the intrinsic rate of the heart (Bers, 2001). HCN channel activity can be reduced during an acidosis by a concurrent reduction in cyclic AMP (cAMP), resulting in a subsequent bradycardia. However, whether similar mechanisms are responsible for the hypercapnic bradycardia displayed by white sturgeon awaits further experimentation.

Previous work with CO_2-sensitive species has shown that myocardial acidosis exerts a negative inotropic effect through H^+/Ca^{2+} competition for binding sites on troponin (Williamson et al., 1976; Gesser and Jørgensen, 1982). White sturgeon have an exceptional capacity for pHi regulation during short term hypercapnia *in vivo* (Baker et al., 2009a), and this could explain preservation of inotropy. Here, the magnitude of the acidosis measured in white sturgeon ventricles exposed to 45 mm Hg PCO_2 (~0.18 pH units; Table 6.2) was less than half of that predicted from its intrinsic buffer capacity (~0.4 pH units; Baker et al, 2009a). Thus, I suggest that some of the protection of white sturgeon cardiac performance *in situ* may be result of the partial pHi compensation observed *in situ*. For example, trout ventricles from hearts exposed *in situ* to low hypercapnia (~9 mm Hg PCO_2) exhibited an acidosis (~0.25 pH units; Milligan and Farrell, 1986) greater than that observed in white sturgeon ventricles (~0.18 pH units) following exposure to 45 mm Hg PCO_2. On the other hand, *P. pardalis* hearts better maintained ventricular pHi *in situ* (Hanson et al., 2009), but were less able to protect inotropy during severe hypercapnia compared to white sturgeon hearts, suggesting the heart of white sturgeon has a greater insensitivity to intracellular acidosis. Inotropic effects due to an acidosis in cardiac muscle tissue have generally been attributed to changes in Ca^{2+} affinity and transport (Williamson et al., 1976; Gesser and Jørgensen, 1982; Shiels et al., 2010), and so further research using ventricular strip preparations to address aspects of inotropic tolerance to pH perturbation is warranted.

6.5.2 Protective effects of adrenergic stimulation on cardiac performance during hypercapnia

White sturgeon hearts are under some degree of adrenergic tone during normocapnic conditions *in vivo* (Crocker et al., 2000), but relatively little is known about their adrenergic

sensitivity. Even so, differences in control cardiac performance between Series 2 and 3 (a 20% lower Q_{max} and PO_{max} in the latter; Fig. 6.3C,D; Fig. 6.4C,D) are unlikely related to tonic levels of perfusate adrenaline (10 and 5 nmol l^{-1} [AD] respectively), as the relative effects of 45 mm Hg PCO_2 were almost identical. Instead, these differences between control values for Q_{max} and PO_{max} may be related to size (2.5 fold larger hearts in Series 3), as larger hearts may lack coronary perfusion, and thus have greater potential limitations in oxygen diffusion in a thicker compact myocardium (Lillywhite et al., 1999). Further studies are needed to describe dose-response effects of adrenaline on cardiac function in sturgeon.

Hypercapnia (22.5 mm Hg PCO_2) induces a persistent (96 h) elevation of plasma adrenaline in white sturgeon (~5-times resting levels; Crocker and Cech, 1998), suggesting an important role for this hormone in ameliorating the negative effects of hypercapnia on cardio-respiratory function. Increased AD can, for example, stimulate I_{Ca} and SR Ca^{2+} uptake, thus increasing cardiac contractility and accelerating relaxation (Bers, 2001). Adrenergic protection of cardiac performance during hypercapnia is commonly observed in fish hearts; high [AD] has been demonstrated to increase inotropy in ventricular strips of both rainbow trout and eel during hypercapnia. Likewise, increasing [AD] (from 5 to 500 nmol l^{-1}) during 45 mm Hg PCO_2 in the present study restored Q_{max} and PO_{max} to control levels by increasing F_V (Table 6.1) and V_S (Fig. 6.4B). Although addition of high concentrations of adrenaline completely protected cardiac performance during hypercapnia, heart rate remained depressed (Fig. 6.4A). Consequently, although Q_{max} and PO_{max} during 45 mm Hg PCO_2 were maintained at control levels, heart function was qualitatively very different than during control conditions.

A lack of effect of adrenaline on f_H was unexpected as AD is known to stimulate HCN channel activity in pacemaker cells, and increase f_H in vertebrate hearts. Additionally, in most fishes examined, adrenergic protection is attributable to increases in both f_H and contractile force. Only rainbow trout hearts working routinely exhibited no increase in f_H in the presence of

high adrenaline (Farrell, 1986), albeit at a much lower CO_2 tension. I speculate that this absence of an effect on f_H may be a direct effect of perfusate pH (6.85 at 45 mm Hg PCO_2) on pacemaker cells rather than pHi effects, as pHi increased in response to elevated AD. This possibility might also explain why hearts exposed to 60 mm Hg PCO_2 (perfusate pH=6.7) exhibited arrhythmia. Thus, while cardiac inotropy may be CO_2-tolerant, negative chronotropic effects may be unavoidable, as no blood pH compensation occurs during exposure to severe hypercarbia (> 45 - 60 mm Hg, Chapter 4). Unfortunately, little is known about the effects of a severe blood acidosis (blood pH decreases of > 0.7) on intrinsic heart rate, as few other vertebrates can tolerate this condition. Cardiac failure as a mechanism of CO_2 toxicity in white sturgeon remains a possibility, particularly considering the presence of a sustained, severe extracellular acidosis.

6.5.3 Conclusions

To place the present study in a broader perspective, various authors (Heisler, 1986; Ultsch, 1996; Brauner and Baker, 2009) have suggested that aquatic hypercarbia has been underestimated as a selective pressure associated with a number of important vertebrate adaptations. White sturgeon display an exceptional tolerance to hypercapnia, and this tolerance extends to cardiac performance. Severe hypercapnia (45 mm Hg PCO_2) only modestly reduced Q_{max} and PO_{max}, and both were restored with adrenergic stimulation or upon return to control CO_2 tensions. Furthermore, this loss of performance was observed in an *in situ* heart preparation, devoid of other possible mediating responses, such as alterations in vagal tone or vascular resistance. The combination of an emergent co-ordinated response (Chapter 3; Baker et al., 2009a; Chapter 4) and inherent CO_2 tolerance of cardiac tissue strongly suggest that CO_2 tolerance may have played an important role in the evolutionary success of sturgeons. Research identifying mechanisms associated with protection of cardiac function during hypercapnia

remains an exciting future direction. As white sturgeon are phylogenetically positioned between elasmobranchs and teleosts, this work may provide important insight into the evolution of CO_2 tolerance in fish hearts.

6.6 Tables

Table 6.1 The effect of hypercapnia (22.5, 45 or 60 mm Hg PCO_2) and maximal adrenergic stimulation (45 mm Hg PCO_2 and 500 nmol l^{-1} [AD]) on rate of ventricular force generation (F_V) in perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. An asterisk indicates a statistically significant difference from normocapnia expose hearts within a given PCO_2 treatment.

		Rate of ventricular force generation (kPa sec^{-1})		
Hypercapnic PCO_2 (kPa)	Number of hearts (n)	Normocapnia	Hypercapnia	Hypercapnia + [AD]
22.5	4[1]	6.5±0.3	7.0±0.2	-
45	7[2]	9.3±0.6	7.7±0.4*	8.8±0.6
60	4[1]	6.1±0.2	5.2±0.3*	-

1. Assessed in hearts from Series 1
2. Assessed in hearts from Series 3

Table 6.2 The effect of hypercapnia (45 mm Hg PCO_2) and maximal adrenergic stimulation (500 nmol g^{-1} [AD]) on white sturgeon ventricular intracellular pH (pHi). *In vivo* values were obtained from ventricles excised from white sturgeons under resting conditions. Control group represents ventricles sampled during 3.75 mm Hg PCO_2. Values are mean ± s.e.m. Letters indicate significant differences among treatments.

CO_2 tension (mm Hg)	Fish number (n)	[AD] (nmol l^{-1})	Perfusate pH (pHe)	Ventricular pH (pHi)
in vivo	8	*in vivo*	7.80±0.01	6.91±0.013[a]
Control (3.75)	2	10	7.80	6.95±0.050[a]
45	4	10	6.85	6.77±0.015[b]
45	8	500	6.85	6.83±0.011[c]

6.7 Figures

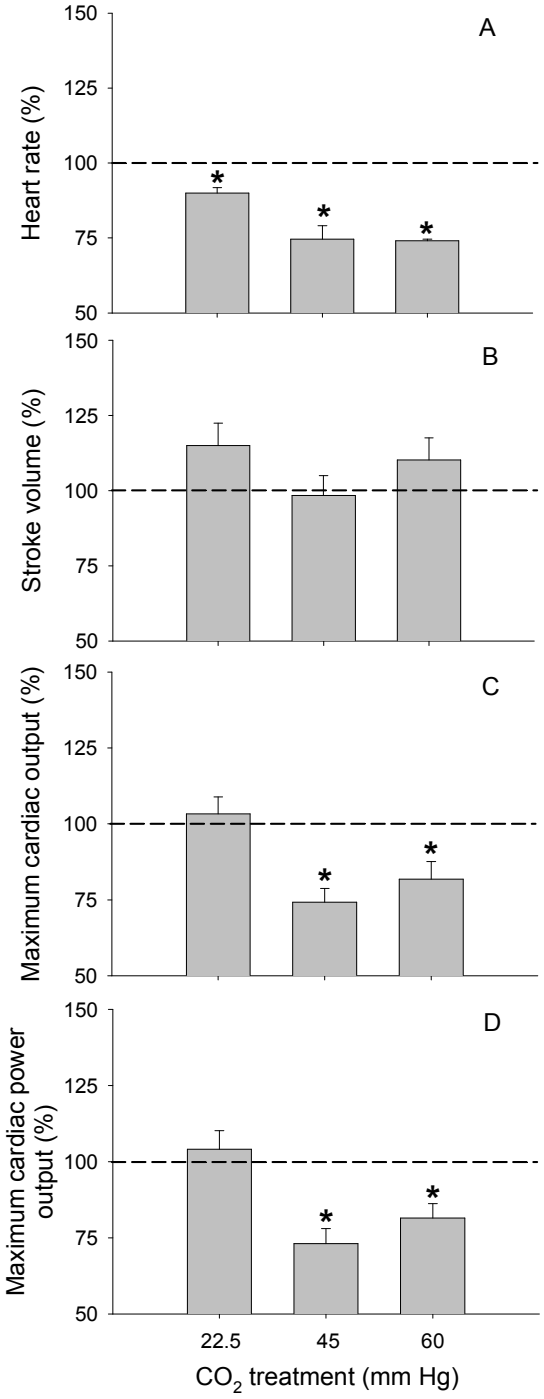


Figure 6.1 The effect of hypercapnia (22.5, 45 and 60 mm Hg PCO_2) on A) heart rate (f_H), B) stroke volume (V_S), C) maximum cardiac output (Q_{max}), and D) maximum cardiac power output, (PO_{max}), expressed as a percentage of control values assessed on perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. An asterisk indicates a statistically significant change from control values within that CO_2 tension. Dotted line represents control values (i.e., 100%) for comparative purposes.

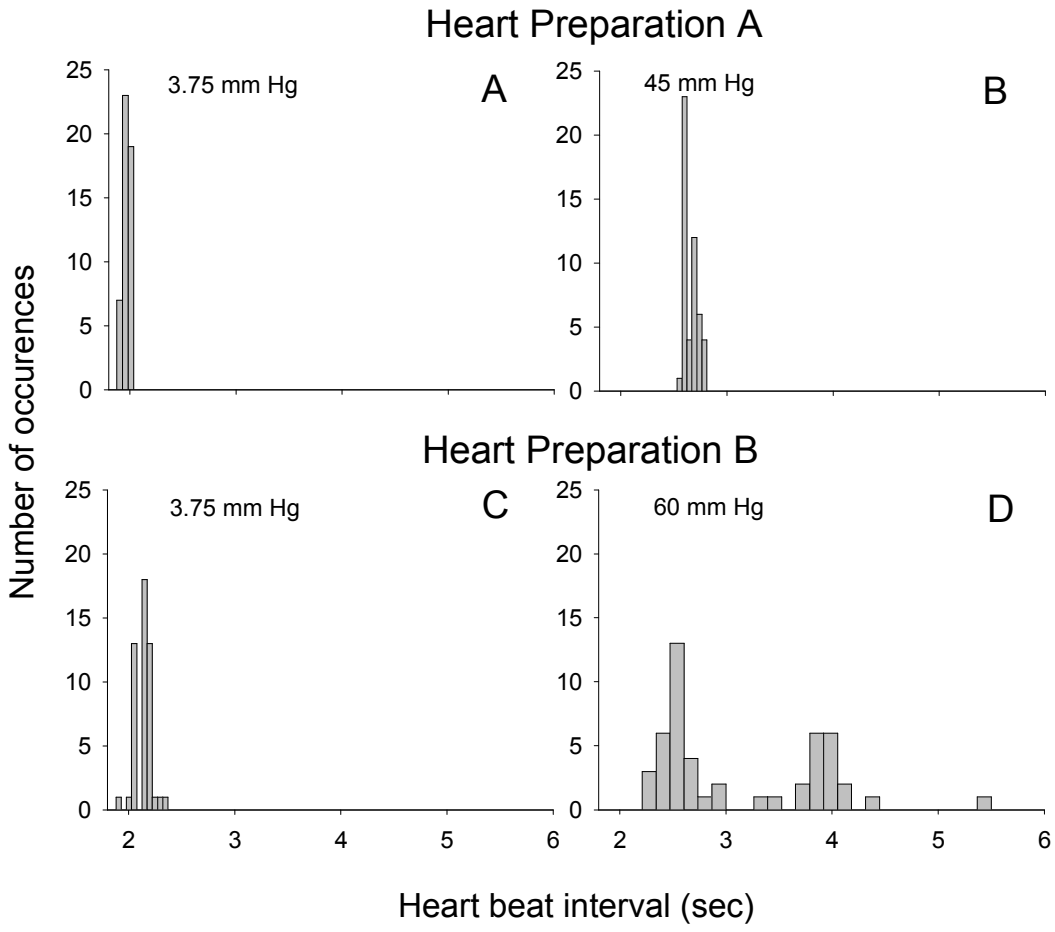


Figure 6.2 A diagram representing the effect of hypercapnia (45 and 60 mm Hg PCO_2) on heart beat interval (time in seconds between beats) during cardiac performance testing. The top two panels (A and B) are data from an *in situ* perfused heart sequentially exposed to A) 3.75 mm Hg PCO_2 and then B) 45 mm Hg PCO_2. The bottom two panels (C and D) are data from an *in situ* perfused heart sequentially exposed to C) 3.75 mm Hg PCO_2 and then D) 60 mm Hg PCO_2. Note the bimodal distribution of long and short heat beat intervals in the heart preparation exposed to 60 mm Hg PCO_2 (Panel D).

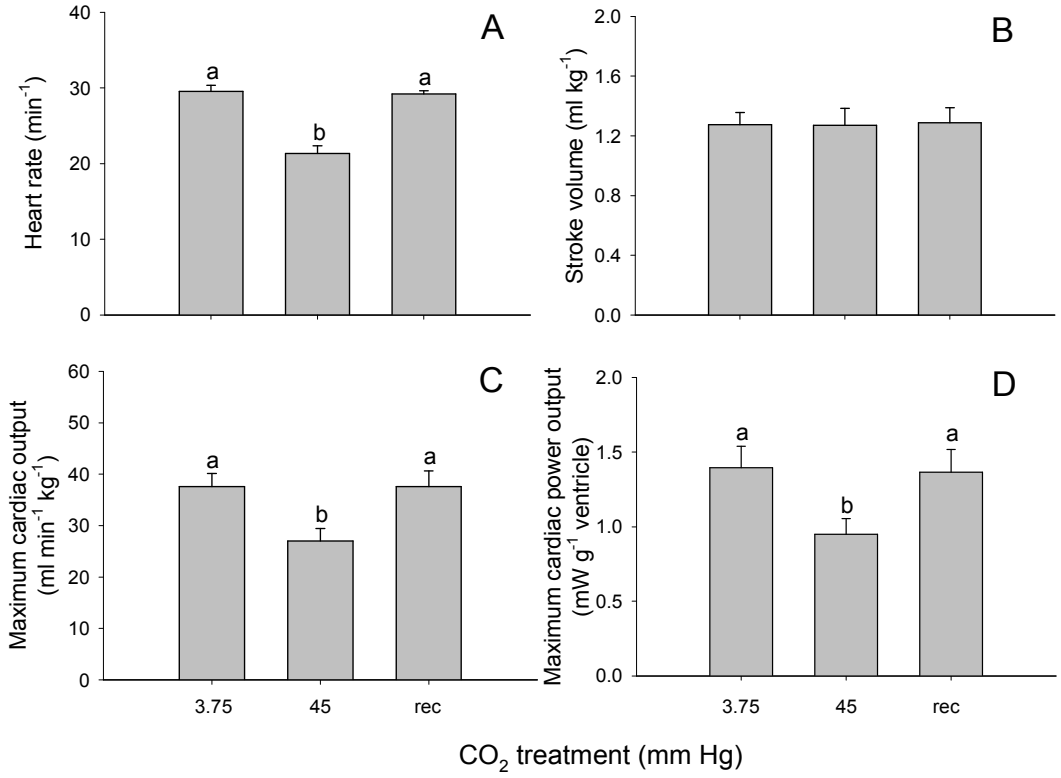


Figure 6.3 The effect of hypercapnia (45 mm Hg PCO_2) and return to control CO_2 tension ("rec"; 3.75 mm Hg PCO_2) on A) heart rate(f_H), B) stroke volume (V_S), C) maximum cardiac output (Q_{max}) and D) maximum cardiac power output (PO_{max}) assessed on perfused white sturgeon hearts *in situ*. Values are means ± s.e.m.(n = 6). Letters indicate statistically significant differences between treatment groups.

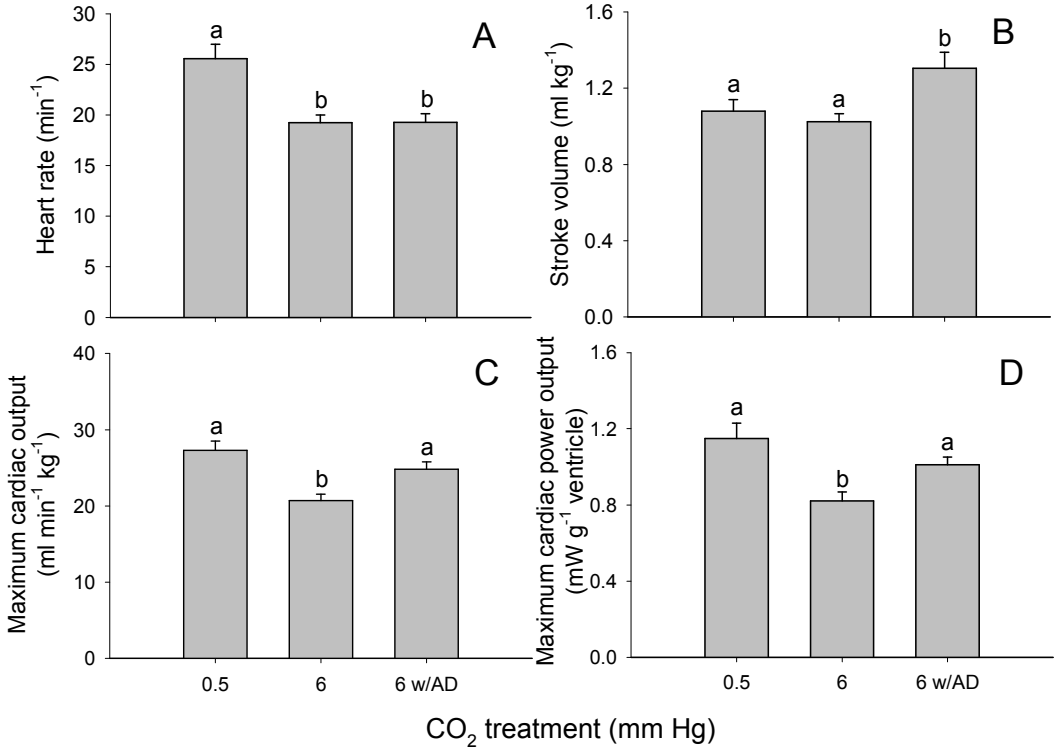


Figure 6.4 The effect of hypercapnia (45 mm Hg PCO_2) in the absence and presence of adrenaline (6 w/AD; 500 nmol l^{-1} [AD]) on A) heart rate, f_H, B) stroke volume, V_S, C) maximum cardiac output, Q_{max}, and D) maximum cardiac power output, PO_{max}, assessed on perfused white sturgeon hearts *in situ*. Values are means ± s.e.m. (n = 8). Letters indicate statistically significant differences between treatment groups.

7: GENERAL DISCUSSION

The typical response of fishes to the respiratory acidosis induced during hypercarbia is branchially driven pHe compensation that is qualitatively and temporally matched by tissue pHi. This thesis examined the hypothesis that CO_2 tolerance in white sturgeon was associated with preferential pHi regulation, (i.e., complete pHi protection during pHe depression). The preceding chapters have clearly demonstrated that CO_2 tolerance is an integrated response involving all levels of organization examined, from behavioural (Chapter 4) to physiological (Chapter 3; Chapter 4; Chapter 5; Chapter 6) to biochemical (Chapter 4; Chapter 5). By incorporating *in vivo*, including NMR (Chapter 5) and traditional physiological investigative (Chapter 3; Chapter 4; Chapter 5) methods, and *in situ* (Chapter 6) techniques, and examining short-term responses to a range of environmentally relevant CO_2 tensions, I have shown that the capacity of white sturgeon to regulate pHi is exceptional. Whether preferential pHi regulation contributes to the enhanced CO_2 tolerance of white sturgeon remains to be determined experimentally, but I hypothesize that it plays an important role. Below, the main findings of the thesis are summarized in light of the initial questions outlined in the introduction. These summaries are followed by a description of the advantages of preferential pHi regulation (including a brief comment on the state of research aimed at identifying mechanisms), and speculation about the phylogeny and ubiquity of preferential pHi regulation in the vertebrate lineage.

7.1 A VALIDATION OF THE TISSUE HOMOGENATE METHOD OF PHI ASSESSMENT FROM TISSUES EXPOSED TO HYPERCARBIA

Interpretation of an acid-base regulatory response at the intracellular level requires accurate measurement of pHi; however, the accuracy of the metabolic inhibitor tissue

homogenate (MITH) method of measuring pHi in fish exposed to elevated CO_2 was previously unknown. Therefore, in Chapter 2, using frozen RBC pellets as a proxy for all tissues, I assessed the effect of CO_2 exposure level on the accuracy of the MITH method. The MITH method provided similar accuracy to previously validated methods even with extremely large changes in CO_2 tensions (up to and including 75 mm Hg PCO_2). In addition, RBC pHi *in vitro*, and calculated intracellular intrinsic buffer capacity during exposure to hypercarbia were consistent with those obtained in other chapters (Chapter 3; Chapter 4). Accurate measures of tissue pHi and estimates of intrinsic buffer values were critical for assessing the role of preferential pHi regulation during aquatic hypercarbia in following chapters.

7.2 PREFERENTIAL PHI REGULATION IS ASSOCIATED WITH CO_2 TOLERANCE

White sturgeon protect tissue pHi (e.g., brain, liver, white muscle and heart) during pHe depression following exposure to moderate and severe short-term hypercarbia (6-48 h of 11.5-45 mm Hg PCO_2; Chapter 3; Chapter 4). Amazingly, this pHi protection extends to CO_2 tensions that ultimately prove lethal (90 mm Hg PCO_2; Chapter 4). Interestingly, during moderate (11-15 mm Hg PCO_2) hypercarbia, white sturgeon also exhibit pHe compensation consistent with strategies typical of teleost fishes. However, as in all other fishes reported to date, white sturgeon were unable to correct for the pHe depression induced by severe ($\geq$ 15 mm Hg PCO_2) hypercarbia.

Thus, white sturgeon exhibit a presently unique combination of efficacious pHe compensation during exposure to low and moderate hypercarbia and exceptional pHi regulation during exposure to hypercarbia in general. Intracellular intrinsic buffer values estimated from homogenized tissue are too low to be responsible for pHi protection implying this preferential pHi regulation is likely due to active pH regulatory mechanisms at the cellular level. I suggest

that preferential pHi regulation could represent the paradigm for CO_2-tolerant fishes. However, preferential pHi regulation has never before been observed in an exclusively water-breathing vertebrate, and so clearly, work on other CO_2-tolerant fishes is necessary to support this premise.

7.3 PREFERENTIAL PHI REGULATION IS NOT ASSOCIATED WITH INCREASES IN WHOLE ANIMAL METABOLIC RATE

Metabolic costs of preferential pHi regulation in white sturgeon may provide insight into evolution of CO_2 tolerance in fishes. By examining organismal $\dot{M}_{O_2}$ and a suite of metabolically-relevant behavioural, physiological and biochemical parameters, I made three novel discoveries. First, as mean $\dot{M}_{O_2}$ increased by 40% at 30 mm Hg PCO_2, but decreased by 30 and 60% at 45 and 60 mm Hg PCO_2 respectively, it is clear that metabolic demands of white sturgeon change greatly according to the severity of hypercarbia to which they are exposed. Second, pHe recovery was often observed concurrent with significant increases in $\dot{M}_{O_2}$, which were not attributable to changes in organismal responses (i.e., ventilation and activity). I suggest that pHe compensation may be associated with increased metabolic demand, some of which could be to support an increase in branchially-driven net acid excretion. This conclusion provides support for the hypothesis that pHe compensation may be limited by metabolic scope.

Lastly, preferential pHi regulation occurred concomitantly with a significant decrease in $\dot{M}_{O_2}$ at 45 and 60 mm Hg PCO_2 in the absence of both biochemical indicators of metabolic suppression (e.g., decreases in rate of liver protein synthesis) and increases in anaerobic respiration (lactate accumulation). Consequently, I hypothesize that preferential pHi regulation during hypercarbia is not associated with a large increase in metabolic demand. Fishes with less metabolically-costly strategies of CO_2 tolerance would have a significant advantage over their

competitors, especially considering that hypercarbic events can occur simultaneously with hypoxia in many aquatic ecosystems. Therefore, preferential pHi regulation may have evolved as a means of coping with hypercarbia without increasing oxygen demand.

7.4 PREFERENTIAL PHI REGULATION IS RAPIDLY ACTIVATED DURING HYPERCARBIA

In the previous chapters (Chapter 3; Chapter 4), complete pHi protection during moderate and severe hypercarbia was observed as early as 6 h. Using NMR technology, *in vivo* pH regulatory and metabolic responses of the heart were recorded in real time during the initial 90 min of exposure to severe hypercarbia to determine 1) how rapidly preferential pHi regulation could be activated and 2) whether this activation was associated with changes in energetic status. White sturgeon hearts did not become acidotic during severe hypercarbia within the time resolution from ^{31}P spectra collected from the heart region of conscious, ventilating white sturgeon. This rapid pHi regulatory response was associated with significant decreases in metabolite concentrations (i.e., [ATP] and [CrP]) relative to their pre-exposed state, but metabolites had recovered by 90 min. Thus, I propose that pHi regulation incurs small and short-lived increases in ATP demand within the first 75 min of exposure to severe hypercarbia. Whether this metabolic cost is due to changes in ATP supply or demand remain to be determined, but it does not appear to be associated with an intracellular acidosis. However, metabolic perturbations do not persist, suggesting metabolic consequences associated with preferential pHi regulation are minor, and this conjecture is supported by whole animal $\dot{M}_{O_2}$ (Chapter 4). Overall, preferential pHi regulation in white sturgeon appears to be a truly rapid response, implying that activation of acid-base regulatory mechanisms responsible for pHi protection is readily achieved. *In vivo* heart pHi regulation of the rapidity and magnitude implied here has not

been previously reported for any other animal species during hypercarbia, indicating a truly remarkable response.

7.5 CARDIAC PERFORMANCE IS EXCEPTIONALLY TOLERANT OF HYPERCARBIA AND ACIDOSIS

Although metabolic costs associated with preferential pHi regulation during hypercarbia appear to be relatively small (Chapter 4; Chapter 5), other consequences of this strategy for CO_2 tolerance may exist. Using an *in situ* heart preparation, I investigated whether the enhanced CO_2 tolerance of white sturgeon came at the expense of maximum cardiac performance (Chapter 6). Although fish hearts are normally regarded as being acidosis intolerant, white sturgeon hearts exhibited exceptional protection of maximum cardiac performance during severe hypercapnia (elevated internal PCO_2): neither maximum cardiac output (Q_{max}) nor maximum cardiac power output (PO_{max}) were reduced at 23 mm Hg PCO_2 , which can be lethal in CO_2-sensitive fish species (Hayashi et al., 2004). Exposure to more severe hypercapnia (i.e., 45 mm Hg PCO_2) significantly reduced heart rate, Q_{max}, PO_{max} and rate of ventricular force generation, and was associated with partial (~55%) ventricular pHi compensation. Even so, these impairments were modest compared to those observed in other CO_2-tolerant species (Q_{max} was reduced ~25% compared to 50% in *P. pardalis* following similar increases in PCO_2; Hanson et al., 2009), and full recovery was possible following a return to control conditions. Adrenergic stimulation during 45 mm Hg PCO_2 returned Q_{max} and PO_{max} to control levels *via* increased inotropy (force of contraction), restoring cardiac scope, but also enhanced ventricular pHi recovery. Plasma adrenaline levels remain elevated *in vivo* in white sturgeon during hypercarbia (Crocker and Cech, 1998), and may play an important role in cardiac function. In contrast, cardiac performance during exposure to hypercarbia severe enough to result in morbidity (i.e., 60 mm

Hg PCO_2) was qualitatively different as *in situ* hearts exhibited arrhythmia at routine input pressures, and a reduction in stroke volume during power assessment. Heart rate reductions were not altered by adrenergic stimulation, and so pHe depression may have direct effects on heart function, even in the absence of a severe intracellular acidosis. Thus, in white sturgeon, exposure to CO_2 levels beyond the limit of organismal survival (i.e., 60 mm Hg) also appear to severely impair cardiac performance.

Taken together, these results demonstrate that the regulatory mechanisms associated with preferential pHi regulation are rapidly activated even *in situ*, in response to CO_2 or exogenous adrenaline, at least in the ventricle. Why heart pHi was not protected *in situ* to the same degree as it was *in vivo* (Chapter 5) remains to be determined. Overall, these findings imply that whole animal CO_2 tolerance may have involved a number of adaptations, including the evolution of acid-insensitive contractile tissue in the heart.

7.6 PREFERENTIAL pHi REGULATION AS A STRATEGY FOR CO_2 TOLERANCE

Most fishes cannot rely on pHe compensation to avoid the acidosis induced by exposure to severe hypercarbia, but must instead be able to cope with or avoid the intracellular acidosis to survive. This ability may ultimately define CO_2 tolerance of a fish. The findings of this dissertation provide clear evidence that white sturgeon avoid an intracellular acidosis through an exceptional capacity for pHi regulation in tissues such as heart and brain. While yet to be investigated extensively, preferential pHi regulation could represent the paradigm for CO_2 tolerant fishes. But what advantages are associated with preferential pHi regulation?

Ignoring for the moment that pHe compensation during severe short-term hypercarbia does not appear to be possible in osmoregulating fishes (Chapter 1, also see Section 7.7 below, for further details), can provide insight into a few of these advantages. Using previously

described solubility and equilibrium coefficients for CO_2 to calculate PCO_2 isopleths in blood and tissues of fish (Boutilier et al., 1984; Brauner et al., 2004; Fig. 1.1), net acid equivalent removal required to recover normocarbic pH within the intra- and extracellular compartments can be estimated (Fig. 7.1). For example, a 1 kg fish (assuming 24% extracellular fluid, 56% intracellular fluid, and the balance non-fluid; see Chapter 3; Chapter 4; Chapter 5) exposed to 7.5 mm Hg PCO_2 (1% CO_2 by volume, and a common experimental challenge) at 12°C would required net uptake of ~4500 μmol of HCO_3^- to recover normocarbic pHe and pHi, of which only ~500 μmol would be intracellular and the remaining ~4000 μmol would be extracellular (Fig. 7.1A). This same fish exposed to 45 mm Hg PCO_2 (6% CO_2 by volume) would required net uptake of ~37,000 μmol of HCO_3^- to recover normocarbic pHe and pHi, of which less than 9000 μmol would be intracellular and ~28000 μmol would be extracellular (Fig. 7.1B). Thus by regulating pHi exclusively, transport of total acid equivalents would be reduced by 88% and 76% at 7.5 and 45 mm Hg PCO_2, respectively. These dramatic differences in net acid equivalent removal between compartments, which are result of lower pHi, lower CO_2 solubility, and higher buffer capacity of intracellular fluids (see Heisler, 1999; Brauner et al. 2004; Brauner and Baker 2009), could translate into substantial energetic savings, if, as the results of Chapter 4 suggest, organismal net acid excretion through presumably branchial sites is metabolically expensive.

There are two other significant challenges associated with moving the acid equivalents associated with pHe compensation during severe hypercarbia. The first is that the highest rates of organismal net acid excretion in fishes are ~1000 μmol kg^{-1} h^{-1}, although in most fishes, rates this high are not achieved or are maintained only briefly (Evans et al., 2005, Brauner et al., 2004; Chapter 3; Baker et al. 2009b). Consequently, the fish exposed to 45 mm Hg PCO_2 in the example above would require at least 37 h to fully return pHe and pHi to normocarbic levels, even if they could maintain these maximal rates, which is unlikely. In contrast, pHi recovery is not limited by rates of branchial net acid excretion, and indeed, a mismatch is created between

cellular and organismal net acid excretion (i.e., blood pH falling below the blood-buffer line, Fig. 3.7, see Section 7.7.1.4 and *A. calva* below) in fish that do preferentially regulate pHi. Thus, pHi protection can be accomplished more rapidly through preferential pHi regulation than through branchially driven pHe compensation.

The second challenge relates to available acid-base relevant counter ions. To maintain electroneutrality within each respective compartment, proton extrusion or excretion from the intra- or extracellular compartment must be accompanied by equimolar anion loss or cation uptake. Intracellularly, net HCO_3^- accumulation is small relative to total ion concentrations (15 mmol l^{-1} at 45 mm Hg PCO_2). In the extracellular compartment however, an increase in [HCO_3^-] to 125 mmol l^{-1} (Chapter 1; Fig. 1.3) is necessary to recover normocarbic pHe during exposure to 45 mm Hg PCO_2, and this accumulation must be matched by either increases in extracellular Na^+ (which would incur severe osmotic challenges, and a final plasma osmolarity of > 400 mosm l^{-1}) or decreases in extracellular Cl^- (which, as plasma [Cl^-] in most fishes is between 90 and 120 mmol l^{-1}, would result in hypochloremia and likely death, Fig 7.1). Therefore, pHi compensation through preferential pHi regulation can be accomplished with relatively less ionic and osmotic perturbation compared to pHi regulation accompanied with pHe compensation.

These three advantages of preferential pHi regulation during hypercarbia (i.e., lower metabolic cost, increased rapidity, and reduced physiological perturbation of pHi compensatory response) could represent the basis through which preferential pHi regulation was selected for. However, the precise trans-membrane acid-base relevant mechanisms responsible for preferential pHi regulation remain to be elucidated. The findings of this thesis provide little support for speculation about pH regulatory mechanisms associated with preferential pHi regulation, as typically the experimental approach for identifying ion transporters employs isolated cell preparations and ion substitution/pharmacological blockers protocols as a first step in classification. Recently, work related to this thesis using isolated hepatocytes from white

sturgeon to identify potential candidates for these mechanisms was undertaken (K. Huynh, D. Baker, R. Harris, J. Church, and C. Brauner, unpublished). These experiments have been met with limited success, as white sturgeon primary hepatocytes previously unexposed to hypercarbia did not compensate for the acidosis induced by hypercarbia. In contrast, primary hepatocytes incubated in severe hypercarbia (45 mm Hg PCO_2) for 24 h regulated pHi back towards levels of normocarbia-exposed cells, but also were able to more rapidly clear an additional acid load induced through ammonia pre-pulse during sustained exposure to severe hypercarbia. Despite this interesting finding, further research is needed to identify which cellular acid extruding/base uptake transporters might be responsible for preferential pHi regulation in white sturgeon.

7.7 EVOLUTIONARY SIGNIFICANCE

White sturgeon represent the most basal vertebrate known to exhibit preferential pHi regulation during hypercarbia. As sturgeon are derived from ancient (i.e., pre-teleost) chondrosteans, they have enormous value for studying vertebrate evolution, including physiological adaptations to the environment (Cech and Doroshov, 2004). Sturgeon characteristics could reflect those of a common ancestor to other vertebrates (Doroshov and Cech 2005), and consequently, research on acid-base physiology in sturgeons may not only address important questions about CO_2 tolerance in fish, but could also potentially provide insight into the evolution of CO_2 tolerance and acid-base regulation in more derived fishes. Because of the difficulties in tracing physiological traits through fossil records, it is unknown whether preferential pHi regulation is an ancestral strategy of primitive fishes. To expand on the significance of the findings presented in this thesis, below I discuss them in an evolutionary context.

7.7.1 Survey of extant primitive fishes

In unicellular organisms, cellular pH regulation is central to tolerating variation in environmental pH or CO_2 levels and thus tight cellular pHi regulation clearly represents the ancestral condition (Booth, 1985). In most multicellular animals that possess gills, characteristics of the blood can be regulated independently of the environment, and the pH of the medium perfusing the cells (i.e., blood and extracellular fluid) is controlled to effectively buffer tissues from acid-base perturbations. Thus, in these animals the first line of defence during an environmental acid-base disturbance is extracellular, with the second line of defence being cellular. Consequently, one could hypothesize that animals which possess the ability to effectively regulate extracellular pH rely on intrinsic buffering to deal with intracellular acid-loading events, and have consequently reduced the capacity to increase active pH regulation intracellularly. In the vertebrate lineage, there is significant support for this hypothesis.

The limited capacity for regulating pHi during a general acidosis is best described in two highly derived but phylogenetically distant vertebrate species, the rat and rainbow trout. In the tetrapod lineage, work on rats has illustrated that during exposure to short-term (hours) hypercarbia and a respiratory acidosis, changes in tissue pHi and pHe are positively correlated (Rothe and Heisler, 1987), although more recent studies show that nervous tissue may begin recovery within that time (60-120 min; Ritucci et al., 1998; Nattie et al., 2002; Kersh et al., 2009). Similarly, fish exhibit a concomitant decrease in pHi and pHe during hypercarbia. In rainbow trout (and most other teleost fish), however, branchial net acid excretion drives pHe compensation which is paralleled by pHi recovery (Fig. 1.2; Wood et al., 1990; Wood and LeMoigne, 1991), although the gill may be an exception (Wood and LeMoigne, 1991). This has been considered to be the typical response for most fishes for over 25 years (Heisler, 1986;

Cameron, 1989; Heisler, 1999; Brauner and Baker, 2009). This relationship between pHe and pHi is clearly dissimilar to that of preferential pHi regulation as observed in the white sturgeon (Fig. 3.9). In fact, very few instances of preferential pHi regulation can be found in the vertebrate lineage, excluding the few tissues mentioned above.

What, then, can be concluded about the possibility of preferential pHi regulation being prevalent throughout the vertebrate lineage? To address this question, I present an examination of what is known about hypercarbia- and hypercapnia-induced acid-base regulation in the Agnathans, Chondrichthyans, Sarcopterygiians, basal (i.e., non-teleost) Actinopterygiians, and the teleosts (summarized in Fig. 7.2). In this way, I provide the foundation for hypotheses regarding the evolutionary significance of preferential pHi regulation in white sturgeon.

7.7.1.1 Agnathans (hagfishes and lampreys)

The hagfishes are the most basal craniates and thus many aspects of their biology may be representative of the common ancestor of vertebrates (Holland and Chen, 2001; Janvier, 2007). Consequently, there is considerable interest in understanding their physiology. Pacific hagfish, *Eptetratus stoutii*, can tolerate severe hypercarbia, and are able to survive 45 mm Hg PCO_2 for 96 h with no morbidity (Baker, Sardella, Rummer and Brauner, unpublished). These animals do not, however, exhibit preferential pHi regulation, as recovery of heart, liver and muscle pHi is qualitatively paralleled by recovery in blood pH (pHe) during aquatic hypercarbia (Baker, Sardella, Rummer and Brauner, unpublished; Fig. 7.3). The CO_2 tolerance of these fishes instead is associated with an exceptional ability to regulate pHe (McDonald et al., 1991; Baker, Sardella, Rummer and Brauner, unpublished; Fig. 7.4), where pHe compensation is able to drive 70% recovery of the 1 pHe unit depression during exposure to 45 mm Hg PCO_2 (Fig. 7.4). The capacity for hagfish to compensate for a respiratory acidosis in the blood compartment is

unmatched among water breathing vertebrates, and may relate to the osmoconforming nature of these fish (Morris, 1965). If their physiology is representative of the ancestral condition, the equimolar Cl^- loss associated with net HCO_3^- accumulation may represent the ancestral compensatory strategy for acid-base regulation during hypercarbia within the vertebrate lineage.

No data on the effects of aquatic hypercarbia on lampreys is currently available. However, sea lampreys, *Petromyzon marinus*, can deal with acid loading in the blood rapidly, and do regulate pHi in RBC during the blood acidosis associated with exhaustive exercise (Tufts, 1991). That said, no link between pHi regulation in RBC and preferential pHi regulation in other tissues has been observed in any fishes. In any case, no evidence of preferential pHi regulation exists in this ancient group of fishes, however further studies should be conducted to confirm this in lampreys.

7.7.1.2 Chondrichthyans (sharks, batamorphs and chimaeriformes)

Some species of elasmobranchs exhibit CO_2-tolerance, such as the starspotted dogfish, *Mustelus manazo*, which can survive 37.5 mm Hg PCO_2 for 72 h without morbidity (Hayashi et al., 2004). Despite this, elasmobranchs do not appear to exhibit preferential pHi regulation during hypercarbia. In the skate, *Raja ocellata*, pHi recovery qualitatively paralleled pHe recovery during aquatic hypercarbia (10 mm Hg PCO_2), although both heart and brain exhibited pHi compensation slightly earlier (at 2 h) than blood (Graham et al., 1990; Wood et al., 1990; Fig. 1.2). During 24 h of exposure to 10 mm Hg PCO_2, *Scyliorhinus stellaris* exhibited similar patterns of HCO_3^- accumulation in extra- and intracellular compartments (Heisler et al., 1988); in contrast, preferential pHi regulation is associated with earlier HCO_3^- accumulation intracellularly (Chapter 3). CO_2 tolerance may be a result of a greater capacity for pHe compensation as in the hagfish above, as plasma [HCO_3^-] in *M. manazo* was ~75 mmol l^{-1} following less than 72 h of

52.5 mm Hg PCO_2 (Hayashi et al., 2004), well above the bicarbonate threshold described in teleosts. As in the Agnathans, there is no evidence to date for preferential pHi regulation, although the chimaeriformes (ratfishes) remain to be investigated.

7.7.1.3 Sarcopterygiians (lungfishes, coelacanth and tetrapods)

All extant lungfishes can tolerate large internal elevations in PCO_2. In episodic air-breathing fishes, a rapid increase in blood PCO_2 is observed in the first minutes following an air breath, and these elevated levels persist until another breath is taken (Graham, 1997). Indeed, all episodic air breathers will experience hypercapnia with breath-holding, and PCO_2 levels may fluctuate between 5 and 31 mm Hg PCO_2 with regularity (e.g., Sanchez et al., 2005). In addition, the seasonal ponds and slow moving rivers these fish inhabit may be prone to hypercarbic events (Heisler, 1982). Thus it is not surprising that the lungfish, *Protopterus dolloi*, can endure severe aquatic and aerial hypercarbia of 37.5 mm Hg PCO_2, or that *L. paradoxa* survives ~50 mm Hg PCO_2 and a blood acidosis for at least 5 h (Amin-Naves et al., 2007). No data relating to acid-base physiology exists for coelacanths. Within the tetrapods, only two salamanders, *Siren lacertina* and *Amphiuma means*, exhibit preferential pHi regulation in heart and white muscle (Heisler et al., 1982) during aquatic hypercarbia. Clearly, much more work is required to investigate acid-base regulation in this most interesting group.

7.7.1.4 Basal actinopterygiians

The extant basal actinopterygiians include the Polypteriformes (bichirs and reedfishes), the Acipenseriformes (paddlefishes and sturgeons), the Lepisosteids (gars) and the Amiiformes (bowfin). The Polypteriforme and the Lepisosteid fishes include extant air-breathing species,

although surprisingly little is known about the acid-base physiology of these fishes (Rahn et al., 1971; Graham, 1997). Why these fishes have not received more attention is unclear, as due to their phylogenetic positions (relative to, for example, teleosts and Sarcopterygiians; Fig. 7.2), the resulting studies could be extremely informative regarding the evolution of fishes (Brauner and Berenbrink, 2007; Frick et al., 2007). Certainly, air-breathing fishes in these groups likely have episodic PCO_2 challenges associated with breath-holding as discussed above for the lungfishes, and some species reside in tropical waterways similar to those in which periodic aquatic hypercarbia occurs (Heisler, 1982). In particular, the reedfish, *Calamoichthys calabaricus*, is known to voluntarily emerge from water onto land, at which time the gills cannot excrete CO_2 effectively (Sacca and Burggren, 1982). Therefore, life history characteristics of these fishes and their natural environment (Graham, 1997; Ilves and Randall, 2005) suggests that they may exhibit CO_2 tolerance. Speculation on the likelihood of preferential pHi in these fishes must await further investigation.

The bowfin, *Amia calva*, is a facultative air breather, exhibiting transient increases in PCO_2 and decreases in blood pH during episodic breathing (Johansen et al., 1970). *A. calva* exhibit relatively high CO_2 tolerance compared to that typically observed in teleosts, surviving 24 h of 45 mm Hg PCO_2 with no morbidity, despite a severe depression in blood pH (~0.75 pH units; Baker and Brauner, unpublished, Fig. 7.5). Exposure to aquatic hypercarbia of 11.5, 22.5 or 45 mm Hg PCO_2 resulted in an initial (within 3 h) blood acidosis greater than that predicted by the intrinsic buffer capacity of the blood, suggesting a contribution of acid equivalents from the intracellular compartment at a rate greater than whole animal net acid efflux. This pattern of pHe depression below the blood buffer line has been observed in *P. pardalis* (Brauner et al., 2004) and the white sturgeon (Chapter 3; Baker et al., 2009a), and is indicative of acid-dumping from tissues, implying activation of cellular pHi regulatory mechanisms. Thus, strong evidence exists for preferential pHi regulation in the bowfin, the only extant species in the Amiiformes,

although the hypothesis that CO_2 tolerance of *A. calva* is associated with preferential pHi regulation is yet to be tested.

With ~30 sturgeon and 2 paddlefish species, the Acipenseriforms contain the most extant species of all the basal actinopterygians. The findings reported here of preferential pHi regulation in white sturgeon have not been confirmed in other species of sturgeons, although a number of sturgeon species tolerate air exposure well (which may result in increased CO_2 tensions internally; Barton et al., 2000). No support for or against preferential pHi regulation in paddlefish is available; paddlefish are, however, recognized as a species easily-reared in aquaculture settings under a variety of environmental conditions, including a wide range of CO_2 levels (van Eenennaam et al., 2004). Nevertheless, I have shown that preferential pHi regulation does occur in at least one species within this group of fishes.

7.7.1.5 Teleosts

Surprisingly, CO_2 tolerance during short-term severe aquatic hypercarbia is not well documented in exclusively water breathing teleosts, but this may be largely due to limited investigation. Only a few studies have unarguably demonstrated short-term CO_2 tolerance in water-breathing teleost fishes. For example, carp survived a rapid increase in PCO_2 to 37.5 mm Hg and a concurrent severe blood acidosis for 96 h (Claiborne and Heisler, 1986). In addition, this study estimated that plasma HCO_3^- accumulation could not account for the total HCO_3^- lost from the water, and assumed the rest of the bicarbonate had been transferred to tissues to compensate for the intracellular acidosis. Thus, carp remain probably the best candidate for preferential pHi regulation among water-breathing teleosts. There is evidence that eel (McKenzie et al., 2003), perch, *Perca flavescens* (D. Baker, and C. Brauner, unpublished), European sea bass, *Dicentrarchus labrax* (Cecchini and Caputo, 2003), Plainfish Midshipman, *Porichthys*

notatus, (Perry et al., 2010), matrinxa (Genus *Brycon*; D. Baker and C. Brauner, unpublished) and some commercially reared fish species may also exhibit CO_2 tolerance, but concluding this is difficult due to conflicting findings between studies, and the variable protocols of CO_2 exposure and methods for assessing tolerance used. Examining whether exceptional pHi protection is associated with CO_2 tolerance in these fish will be extremely informative in determining the ubiquity of preferential pHi regulation in the teleost group.

The existence of preferential pHi regulation during aquatic hypercarbia has been established in only a few teleosts to date, and these fishes are all facultative air breathers. For example, during air-breathing induced hypercapnia, *Synbranchus marmoratus* experienced an increase in blood PCO_2 (to 26 mm Hg) and decrease in blood pH (0.5 pH units) for 4 days, but regulated pHi in heart and white muscle at normocapnic levels. *P. pardalis* also preferentially regulates tissue pH during aquatic hypercarbia and a severe blood acidosis; even when fish were exposed to 45 mm Hg PCO_2, heart, liver and white muscle pHi were not significantly different from pHi in control tissues. Therefore, although preferential pHi regulation does occur in teleosts, it may be that it is only exhibited by those that regularly experience transient internal PCO_2 elevations, as occurs in facultative air-breathing fishes.

7.7.2 The origin of preferential pHi regulation

Whether preferential pHi regulation evolved prior to or following the divergence of the actinopterygiians and sarcopterygiian lineages is currently unknown. From the limited data set above, I will now discuss three ideas relating to the origin of preferential pHi regulation. I postulate that preferential pHi regulation evolved to enhance survival during aquatic hypercarbia in early Osteichthyans. I further speculate that the origin of preferential pHi regulation was associated with the advent of two key vertebrate events, the origin of osmoregulation, and the

invasion of freshwater. Finally, I hypothesize that preferential pHi regulation may have been a necessary exaptation for the evolution of air-breathing in the vertebrate lineage.

Agnathans and Chondrichthyans both rely on their great capacity for pHe compensation, and not on preferential pHi regulation, for enhanced CO_2 tolerance. In hagfish and elasmobranchs, HCO_3^- accumulation (an indicator of net acid excretion) appears to be more rapid and substantially greater (70-80 mmol l^{-1}) – well beyond the proposed bicarbonate threshold of 27-33 mmol l^{-1} – than that of the phylogenetically distinct Osteichthyan fishes (hagfish, Baker, Sardella, Rummer and Brauner, unpublished, Fig. 7.4; elasmobranchs, Hayashi et al., 2004; Wood et al., 1990; Fig. 1.2A). This enhanced pHe compensatory response may be related to a greater availability of acid-base relevant counter ions (i.e., Na^+ and Cl^-) for counter ion exchange than in the osmoregulating Osteichthyans (Section 7.6; see Brauner and Baker, 2009). The capacity and rapidity of pHe compensation in these ancestral fishes may have partially obviated the need for tight regulation of tissue pHi in response to hypercarbia.

In contrast, all Osteichthyans are osmoregulators (plasma osmolarity ~300 mOsm l^{-1}; plasma $[Cl^-]$ ~100 mmol l^{-1}), and so have much less Cl^- available for net exchange for HCO_3^- than either hagfishes (plasma $[Cl^-]$ ~500 mmol l^{-1}) or Chondrichthyans (plasma $[Cl^-]$ ~225 mmol l^{-1}). Although other limitations may affect pHe compensation (see below), it is clear that in adopting an osmoregulatory strategy, early vertebrates would have experienced a significant reduction in their capacity for pHe compensation. Therefore, exposure to short-term aquatic hypercarbia at levels requiring HCO_3^- accumulation greater than the previously described bicarbonate threshold (i.e., > 15 mm Hg PCO_2; Heisler, 1986) would likely have required additional pH compensatory response for survival. This may have provided the selective pressure for preferential pHi regulation.

Aquatic hypercarbia may have been a common challenge for early Osteichthyans, especially in the first hyposaline or freshwater environments available for invasion by fishes.

Certainly, current tropical ecosystems are known to experience daily fluctuations in CO_2, due to high biomass producing CO_2 at a rate greater than that of diffusive loss of CO_2, from the water surface – CO_2 tensions greater than 60 mm Hg have been measured (Chapter 1; Heisler et al., 1982; Ultsch, 1996). The first freshwater available for exploitation by fishes may also have contained significant plant biomass, and similar PCO_2 profiles (Ultsch, 1996). Therefore, fishes residing in these waters for food or shelter would have required high CO_2 tolerance. However, these freshwater environments may have provided challenges to branchially-driven pHe compensation. For example, freshwater contains much lower [HCO_3^-] which can limit rates of branchial net HCO_3^- uptake (Heisler, 1999). In addition, freshwater is poorly buffered relative to seawater (Heisler, 1999), and consequently, small elevations in PCO_2 have greater effects on freshwater pH, which can limit branchial acid-base relevant transport (Lin and Randall, 1993). These factors may have contributed to the absence of a pHe compensatory response during aquatic hypercarbia in some Amazonian fish species (Brauner et al., 2004; D. Baker and C. Brauner, unpublished). While the relative timing of vertebrate freshwater invasion and the origin of osmoregulation in Osteichthyans is controversial, either event might have contributed to, or been associated with, the evolution of preferential pHi regulation.

Was preferential pHi regulation an exaptation for air breathing? Undoubtedly, CO_2 tolerance is a pre-requisite for facultative air-breathing, as episodic PCO_2 elevations accompany air-breathing during either air emersion (due to reduced CO_2 excretion rate at the gill, Graham, 1997) or aquatic hypoxia (associated with immersion between breaths, or breath holding, Graham, 1997). Consequently, CO_2 tolerance might have been critical for early air-breathing vertebrates as well, as the effect of changes in PCO_2 on pH is relatively greater at low CO_2 tensions (see Fig. 1.1, 3.9). Assuming PCO_2 in the blood of the first air-breathing fishes was similar to water breathers (2-4 mm Hg PCO_2), air breathing could have induced a significant acidosis that would have occurred too rapidly for compensation through branchial mechanisms

(i.e., pHe regulation). Thus, preferential pHi regulation might have been highly advantageous for these fishes. This hypothesis is supported by the observation that the few fish species other than white sturgeon that exhibit preferential pHi regulation are facultative air breathers (*S. marmoratus*, Heisler, 1982; *P. pardalis*, Brauner et al., 2004; possibly *A. calva*, Brauner and Baker, 2009). Among the Osteichthyans, only the Acipenseriformes and the Coelocanthiformes contain no extant air-breathing species. Should preferential pHi regulation be found to be prevalent among the basal actinopterygiians, the observation that white sturgeon exhibit this response would provide support for the hypothesis that it was an exaptation for air-breathing.

As a final note, speculation based on the limited number of extant non-teleost Osteichthyan fish species (~60 extant species in total) should be considered with appropriate caution. Drawing conclusions about evolutionary trends (such as those regarding the origin of preferential pHi regulation) from such a poorly represented, albeit extremely important (Brauner and Berenbrink, 2007), group is beyond the scope of this thesis. However, the work described in this thesis represents a substantial contribution to our understanding of strategies of acid-base regulation during aquatic hypercarbia in a primitive CO_2-tolerant fish species, and so provides a platform on which hypotheses can be developed to address questions about the evolution of acid-base physiology in vertebrates.

Clearly a great deal of work remains to test the hypothesis that preferential pHi regulation evolved in response to a) the trade-offs in pHe compensation associated with the origin of osmoregulation and b) the limitations to pHe compensation imposed by exploitation of newly created freshwater environments. However, if validated, these ideas may also support Ultsch's (1996) proposal that freshwater hypercarbia has been overlooked as a parameter influencing the vertebrate transition of life from water to land.

7.7 FINAL THOUGHTS

This thesis provides contributions to our understanding of CO_2 tolerance and survival during aquatic hypercarbia in fishes, yet much remains to be discovered. *In vivo* tissue pHi regulation as seen in these animals is currently unmatched in the vertebrate world, and, as I suggest, likely plays a prominent role in survival. Recent experiments on isolated hepatocytes from white sturgeon have demonstrated a pHi regulatory response to a large elevation in PCO_2 (45 mm Hg) thus supplying an avenue through which mechanistic questions can be addressed (K. Huynh, D. Baker, R. Harris, J. Church , and C. Brauner, unpublished). Future research describing the specific mechanisms associated with preferential pHi regulation remains an exciting area for exploration. The high CO_2 tolerance observed in sturgeons throughout these experiments probably underestimates what sturgeons would survive in the wild, as ecologically relevant hypercarbic events would have a more gradual (e.g., hours) onset, an episodic nature, and the highest levels of CO_2 would only occur briefly. Consequently, white sturgeon appear to be extremely well adapted to environments prone to severe hypercarbic challenges, despite the unlikelihood of this challenge occurring within their current distribution of rivers, estuaries and the Pacific Ocean. Given this, it is unknown why sturgeons are so hypercarbia tolerant. Whether it is just a characteristic of a hardy fish or is a trait that evolved early in its evolutionary history specifically in response to CO_2 remains a mystery.

7.9 Figures

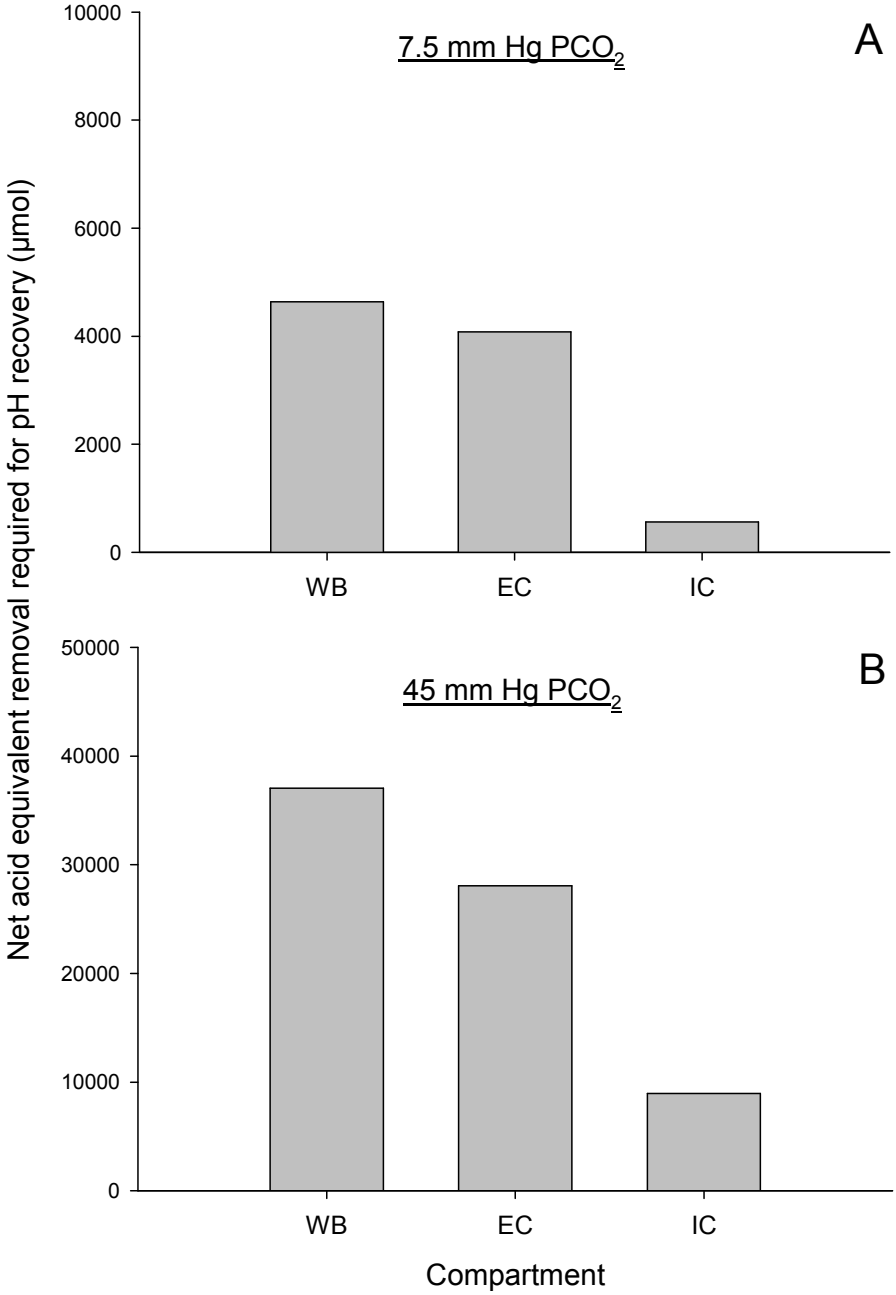
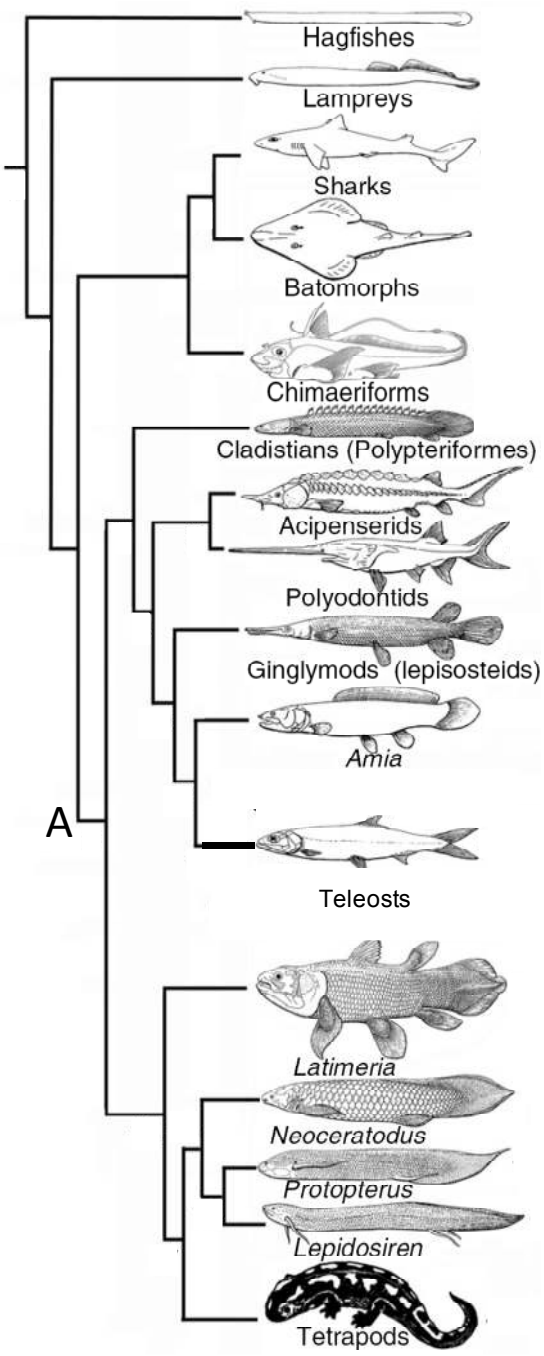


Figure 7.1 Net acid equivalent removal required to recover normocarbic pH in the whole body (WB) (i.e., extra- and intracellular compartments), extracellular (EC), or intracellular (IC) compartments ofa 1 kg fish at 12°C during exposure to A) 7.5 mm Hg PCO_2 and B) 45 mm Hg PCO_2. In B, whole body total chloride ion content (WB Cl^-) is also indicated to illustrate counter ion exchange limitations. CO_2 solubility and equilibrium constants are calculated from previously determined equations (Boutilier et al., 1984). Note differing scales on y-axis between A and B.

Figure 7.2 A summary of acid-base relevant physiological and behavioural characteristics overlaid on a phylogenetic representation of the interrelatedness within the craniate lineage, using the topology that is most widely accepted by morphologists and palaeontologists. Within each taxon, "CO_2 tolerance" refers to whether there are examples of fishes that can survive exposure to severe (>15 mm Hg PCO_2) hypercarbia, "air breathing" refers to whether there are examples of air breathing fish species, and "preferential pHi regulation" refers to whether complete pHi protection during severe pHe depression has been observed in any species. A dash "—" indicates no data exist for this group, and an "i.e." indicates indirect evidence exists for this category (see text for details). Phylogeny modified from Janvier, 2005.



		CO_2 tolerance	Air breathing species	Preferential pHi regulation
Hagfishes	osmoconformers	yes	no	no
Lampreys	osmoregulators	—	no	—
Sharks	osmoconformers	yes	no	no
Batomorphs	osmoconformers	—	no	no
Chimaeriforms	osmoconformers	—	—	—
Cladistians (Polypteriformes)	osmoregulators	i.e.	yes	i.e.
Acipenserids	osmoregulators	yes	no	yes
Polyodontids	osmoregulators	—	no	—
Ginglymods (lepisosteids)	osmoregulators	i.e.	yes	—
Amia	osmoregulators	yes	yes	i.e.
Teleosts	osmoregulators	yes	yes	yes
Latimeria	—	—	—	—
Neoceratodus	osmoregulators	i.e.	yes	—
Protopterus	osmoregulators	yes	yes	—
Lepidosiren	osmoregulators	yes	yes	—
Tetrapods	osmoregulators	yes	yes	yes

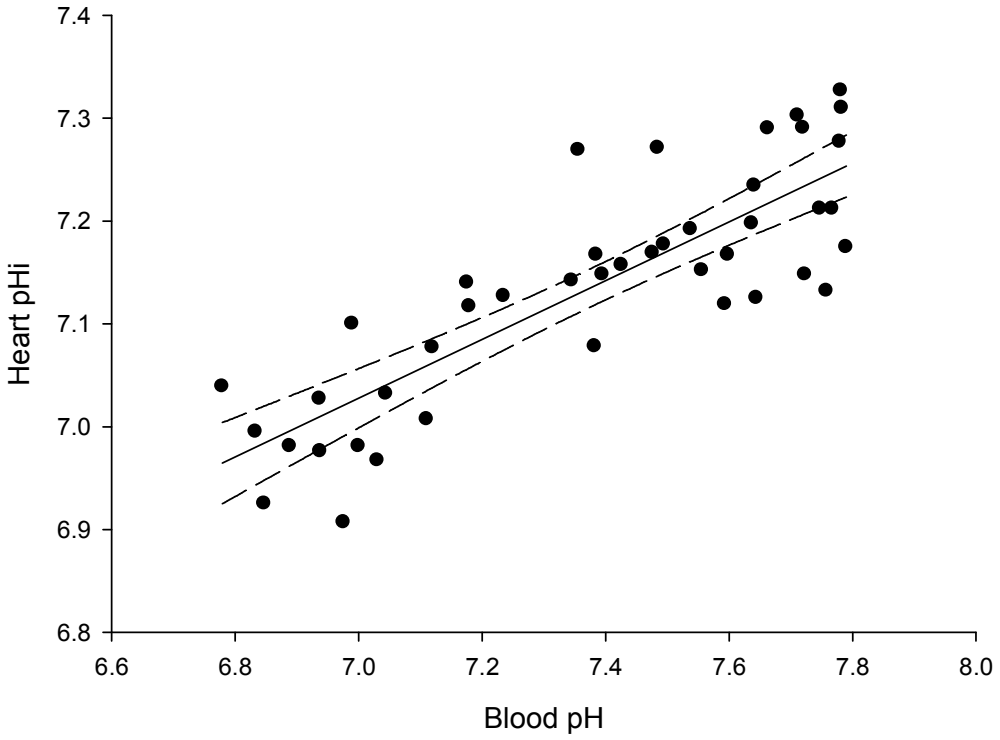


Figure 7.3 The relationship between blood pH (pHe) and intracellular pH (pHi) of heart following recovery during exposure to short-term (3-96 h) hypercarbia (30 and 45 mm Hg PCO_2) in Pacific hagfish. Each point represents a single animal. Dotted lines represent 95% confidence intervals. Blood pH and heart pHi are significantly correlated ($p < 0.001$, $m = 0.29$, $r^2 = 0.71$)

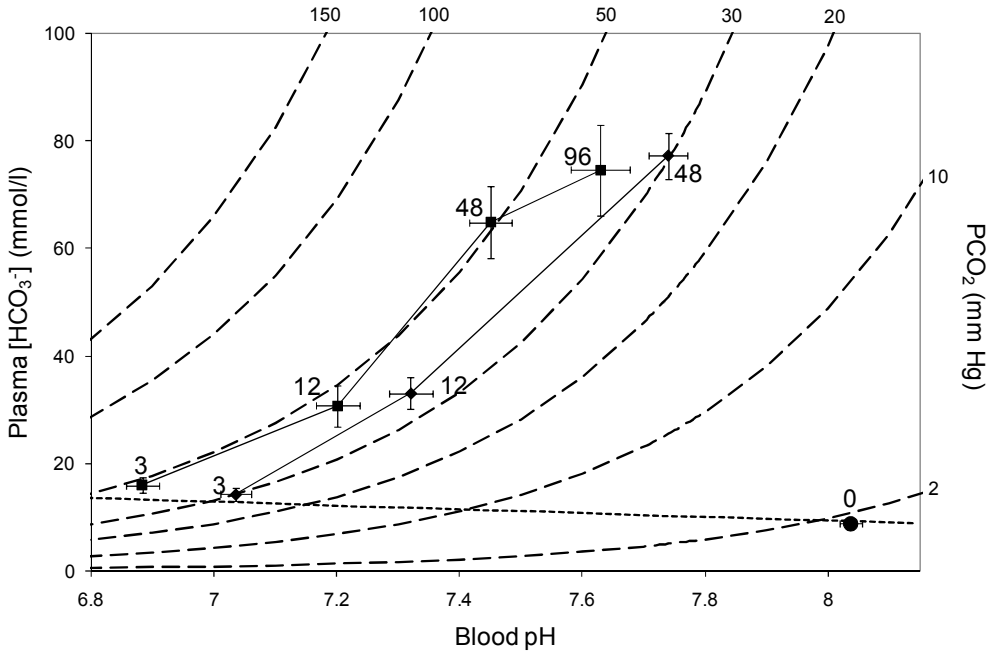


Figure 7.4 The effect of short-term (96 h) hypercarbia (30 and 45 mm Hg PCO_2) on blood pH (pHe) and plasma [HCO_3^-] in Pacific hagfish as represented on a pH/HCO_3^-/CO_2 plot. Values are means ± s.e.m. (n = 6-8). Note plasma [HCO_3^-] are observed much greater than the 27-33 mmol l^{-1} threshold describe in teleosts (see Chapter 1 for more details). Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point represent exposure time. The dotted line indicates intrinsic buffer value of whole blood. Data and buffer values from Baker, Sardella, Rummer and Brauner, unpublished.

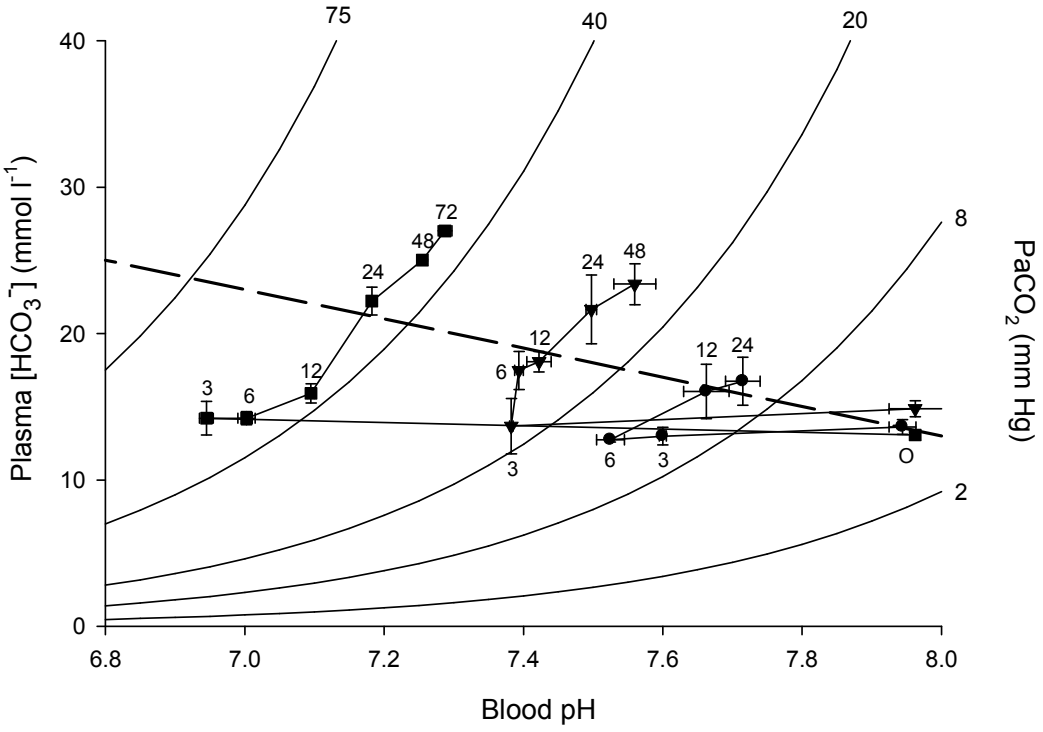


Figure 7.5 The effect of short-term (24-72 h) hypercarbia (11.5, 22.5 and 45 mm Hg PCO_2) on blood pH (pHe) and plasma HCO_3^- in *Amia calva* as represented on a pH/HCO_3^-/CO_2 plot. Values are means ± s.e.m. (n = 3). Note data points fall below the blood buffer line during early (3 h) exposure to hypercarbia, indicating the contributions of acid equivalents to the blood, presumably from the intracellular compartment (although not RBC) (see text for details). Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). Numbers proximal to each data point represent exposure time. The dotted line indicates the non-bicarbonate (i.e., intrinsic) buffer value of whole blood. Data and buffer values from Baker and Brauner, unpublished.

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