VERTEBRATE PREFERENTIAL INTRACELLULAR PH REGULATION DURING SEVERE ACUTE HYPERCARBIA

by

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Abstract

Environmental CO_2 tensions reach >8 kPa (*ca*. 79,000 μatm; hypercarbia) in some habitats and create severe acid-base challenges for vertebrates. Typically, during a hypercarbic-induced respiratory acidosis, changes in blood pH are compensated for, which returns pH to its normal value, and this is coupled to tissue pH (pH_i) regulation. However, during acute environmental CO_2 exposure, this process may be limited to <2 kPa PCO_2. Some fishes fully protect tissue pH (pH_i) (preferential pH_i regulation) despite large sustained reductions of pH_e (>1 pH unit) and can tolerate PCO_2 >3 kPa. I hypothesized that preferential pH_i regulation is used by adult fishes and embryonic amniotes during severe acute acid-base disturbances. This was investigated by examining (1) whether preferential pH_i regulation is a general response to various types of acid-base disturbances, (2) surveying fishes for the presence or absence of preferential pH_i regulation, and (3) whether preferential pH_i regulation is used during development in reptiles.

Using white sturgeon, I found that preferential pH_i regulation is not a general response to both respiratory and metabolic acidoses. Despite a robust capacity for preferential pH_i regulation during respiratory acidoses, not all tissues were protected during metabolic acidoses to the same degree. Preferential pH_i regulation was observed to be a common pattern of acid-base regulation amongst fishes in response to severe acute hypercarbia. A total of 20 species, ranging from basal ("primitive") to derived, were examined and 18 were observed to use preferential pH_i regulation. Finally, developing amniotes (snapping turtle and American alligator) used preferential pH_i regulation during severe acute respiratory acidosis, but the capacity for pH_i regulation was progressively reduced throughout development.

This thesis demonstrates that preferential pH_i regulation is likely a common strategy of acid-base regulation occurring in response to severe acute hypercarbia in adult fishes and possibly amniotes. I propose that preferential pH_i regulation is an embryonic vertebrate strategy, that has been retained or lost in adults depending on the environmental acid-base challenges they face.

Lay Summary

Acid-base homeostasis in vertebrates can be disrupted by high environmental CO_2 (hypercarbia), which creates severe acid-base disturbances. Some vertebrates are exceptionally hypercarbic, likely due to their ability to tightly protect tissue pH (pH_i) despite a reduction in extracellular pH (termed preferential pH_i regulation). My thesis explores preferential pH_i regulation in vertebrates across phylogenies and during development in response to severe acute hypercarbia. A survey of 20 fish species showed that preferential pH_i regulation is used by 18 of these species; it is also used during severe acute hypercarbia in reptilian embryos. These findings suggest preferential pH_i regulation is a common vertebrate pattern of pH regulation, possibly arising in embryos and retained or lost in adult vertebrates depending on their environment; this may have been important for major evolutionary transition in vertebrates, including the evolution of air breathing and the transition from life in water to life on land.

Preface

A version of Chapter 2 has been published. Shartau, R. B., Baker, D. W., and Brauner, C. J. (2017). White sturgeon (*Acipenser transmontanus*) use different strategies for pH regulation depending on the type of acid-base disturbance. *Journal of Comparative Physiology B*. 187:985-994. I designed the experiments, collected and analyzed the data, and wrote the manuscript with assistance from Daniel Baker and under supervision from Colin Brauner.

Chapter 3 was a collaborative project with Baker, D. W., Harter, T. S., Aboagye, D. L., Allen, P. J., Val, A. L., Crossley II, D. A., Kohl, Z. F., Hedrick, M. S., and Brauner, C. J. Preferential intracellular pH regulation may contribute to fish diversity in severely hypercarbic habitats. I designed the experiments with input from Colin Brauner. I setup the experiments and collected the data with assistance from Daniel Baker, Till Harter, Daniel Aboagye, Peter Allen, Adalberto Val, Dane Crossley, Zachary Kohl and Michael Hedrick. I analyzed the data and wrote the manuscript under supervision from Colin Brauner.

A version of Chapter 4 has been published. Shartau, R. B., Crossley II, D. A., Kohl, Z. F., and Brauner, C. J. (2016). Embryonic common snapping turtles (*Chelydra serpentina*) preferentially regulate tissue pH during acid-base challenges. *Journal of Experimental Biology*. 219(13): 1994-2002. I designed the experiments along with Dane Crossley and Colin Brauner. I collected the data with assistance from Dane Crossley and Zachary Kohl. I analyzed the data and wrote the manuscript under supervision from Colin Brauner.

A version of Chapter 5 has been published. Shartau, R. B., Crossley II, D. A., Kohl, Z. F., Elsey, R. M., and Brauner, C. J. (In press). American alligator (*Alligator mississippiensis*) embryos tightly regulate intracellular pH during a severe acidosis. *Canadian Journal of Zoology*. I designed the experiments along with Dane Crossley and Colin Brauner. I collected the data with assistance from Dane Crossley and Zachary Kohl. Ruth Elsey supplied the animals. I analyzed the data and wrote the draft under supervision from Colin Brauner.

The experiments in the thesis followed protocols that were approved by the UBC animal care committee (animal care no: A11-0235).

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List of Symbols and Abbreviations

βNHE	β-adrenergic Na^+/H^+ exchanger
AE	Anion exchanger
ENaC	Epithelial Na^+ channel
Hb	Hemoglobin
HC13	Hypercarbic hypoxia (13kPa PCO_2, 9kPa PO_2)
HC3.5	Hypercarbic (3.5kPa PCO_2, 21kPa PO_2) condition
Hct	Hematocrit
MCT	Monocarboxylate transporter
kPa	kilo Pascal, a unit of pressure
mM	millimolar
MRC	Mitochondrion rich cell (ionocyte)
NBC	Na^+/HCO_3^- co-transporter
NC	Normocarbic (0.03kPa PCO_2, 21kPa PO_2) condition
NHE	Na^+/H^+ exchanger
PCO_2	Partial pressure of CO_2
pH	$-\log[H^+]$
pH_e	Blood (extracellular) pH
pH_i	Tissue (intracellular) pH
pK’	Apparent negative log of dissociation constant (dependent on temperature and ionic strength)
PO_2	Partial pressure of O_2
RBC	Red Blood Cell
s.e.m.	Standard error of the mean
VHA	V-type H^+-ATPase

Glossary

Coupled pH regulation	pH_i changes in a qualitatively similar fashion as pH_e
Exaptation	An adaptation that has been co-opted for another, unrelated use
Hypercapnia	Elevated internal CO_2
Hypercarbia	Elevated environmental CO_2
Hypochloremia	Reduced level of chloride ions in the blood
Metabolic acidosis	Reduced pH because of a reduction in HCO_3^- at a constant PCO_2
pH compensation	The process of pH recovery involving one or more mechanisms
pH recovery	A return of pH to its normal value following an acid-base disturbance
Preferential pH_i regulation	pH_i is regulated independently of pH_e
Respiratory acidosis	Reduced pH because of increased blood CO_2 from an environmental or internal source

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Chapter 1: General Introduction

The overall goal of my thesis was to examine the strategies of vertebrate acid-base regulation in response to severe acute acid-base disturbances, predominantly induced by exposure to elevated environmental CO_2 (hypercarbia). Specifically, this thesis explores preferential intracellular pH regulation, a pattern of acid-base regulation that is markedly different from what has previously been considered the typical vertebrate pattern (Brauner and Baker, 2009; Shartau et al., 2016a). Typically, vertebrates exposed to severe hypercarbia experience a large rapid reduction in both extracellular pH (pH_e) and intracellular pH (pH_i), and compensation of pH_i is dependent on partial compensation of pH_e (Fig. 1.1); this is referred to as coupled pH regulation (Shartau et al., 2016a). In contrast, a few vertebrates are able to preferentially regulate pH_i despite large uncompensated extracellular acidoses during exposure to severe hypercarbia (Fig. 1.2) (Shartau et al., 2016a), a phenomenon that is poorly understood and had only been observed in three fishes and one aquatic tetrapod prior to this dissertation. Using a diverse selection of species ranging from basal to derived fishes, and amniotes, this thesis explores preferential pH_i regulation in vertebrates across phylogenies and during ontogeny. My overall hypothesis for this thesis is that preferential pH_i regulation is a widely used strategy amongst vertebrates to maintain pH homeostasis during severe acute acid-base disturbances. Based on this hypothesis, it is predicted that preferential pH_i regulation will: (1) confer protection against different types of severe acute pH disturbances, (2) be a widely used pattern of pH regulation amongst vertebrates, and (3) confer pH_i protection in animals unable to acutely utilize coupled pH regulation.

This General Introduction will review what is presently known about acid-base regulation in vertebrates in relation to coupled pH regulation and preferential pH_i regulation. The challenges associated with acid-base regulation during hypercarbia will be explored, the putative origins of preferential pH_i regulation are discussed and finally, the objectives and organization of the subsequent data chapters are provided.

1.1 Acid-base regulation in vertebrates

It is well known that absolute physiological pH values differ between species, differ between body compartments within species and are affected by temperature (Rahn, 1974); however, within a given system, pH values are regulated within a relatively narrow range (Cameron, 1989a; Heisler, 1984). Deviations from normal physiological pH values can affect molecular charge, altering the structure and function of proteins, lipids, carbohydrates and nucleic acids, and, ultimately, reducing whole-animal performance (e.g. reduce heart and skeletal muscle contractility, alter metabolic pathways, and disrupt cellular signalling and processes such as volume regulation) (Occhipinti and Boron, 2015; Putnam and Roos, 1997). The degree to which a pH change affects function depends on the system in question. Disturbances to acid-base homeostasis may arise from respiratory or metabolic sources.

Respiratory acidoses occur due to an increase in blood CO_2, either from the environment (hypercarbia) or by retention of metabolically produced CO_2 (hypercapnia). Typical arterial PCO_2 values for adult water and bimodally breathing fishes, reptiles and mammals are 0.1–0.5 (Ultsch, 1996), 0.5–3.5 (Shartau and Brauner, 2014), 1.8–4.3 (Ultsch, 1996) and 4.5–5.6 kPa PCO_2 (Arieff et al., 1976; Malan et al., 1985; Wood and Schaefer, 1978; Yaksh and Anderson, 1987), respectively. Any increase in arterial PCO_2 beyond those values shifts the equilibrium of the CO_2 hydration reaction ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$), promoting the formation of H^+ and HCO_3^-, thus lowering pH and resulting in acidosis.

Metabolic acidoses occur due to the production of metabolically generated acid, which lowers HCO_3^- at relatively constant PCO_2 (Occhipinti and Boron, 2015); often metabolic acidoses occur alongside respiratory acidoses (mixed acidosis) (Kieffer et al., 1994; Wang et al., 1994). In fishes, compensation of a metabolic acidosis is primarily dependent on net exchange of acid-base relevant ions at the gills (Evans et al., 2005; Hwang et al., 2011), and to a lesser degree, excretion of acidic equivalent in the form of titratable acidity and ammonium ions through renal pathways (Kwong et al., 2014).

During an acute respiratory acidosis, reductions in pH_e are associated with qualitatively similar reductions in pH_i. Following the onset of a respiratory acidosis, the

compensation of pH_i is often more rapid than that of pH_e, but complete correction of pH_i generally requires pH_e compensation of >50% (Shartau et al., 2016a). This is referred to as 'coupled pH regulation' whereby changes in pH_i are coupled to changes in pH_e. Vertebrates relying on pH_e regulation are considered to use coupled pH regulation to maintain acid-base homeostasis during an acute persistent acidosis; this is the most widely observed response both *in vivo* and *in vitro* that had been observed prior to this thesis (Shartau et al., 2016a).

1.1.1 Extracellular compartment

Acid-base disturbances in vertebrates can be minimized or compensated by either (i) direct transfer of acid-base relevant ions between the cell and blood, and/or the blood and the environment, (ii) buffering with bicarbonate and non-bicarbonate buffers, or (iii) altering ventilation rate to modify blood PCO_2 and, thus, pH via the CO_2-HCO_3^- buffer system (Brauner and Baker, 2009; Evans et al., 2005; Heisler, 1984). The primary mechanism of short-term acid-base compensation in terrestrial air breathers consists of the latter because blood PCO_2 is high relative to environmental levels (e.g. ~5 kPa vs. <0.1 kPa PCO_2, respectively), so considerable adjustment of pH_e can be accomplished through changes in ventilation; thus the buffering power of the CO_2-HCO_3^- system is large in these animals (Occhipinti and Boron, 2015). In water breathers this mechanism is much less effective due to the low blood PCO_2 levels (~0.3-0.7 kPa vs. <0.1 kPa PCO_2, respectively) and similarity to environmental levels (Heisler, 1984). Thus, water breathing fishes rely on buffering to minimise acid-base changes, and direct transfer of acid-base relevant ions to compensate acid-base disturbances (Brauner and Baker, 2009; Perry and Gilmour, 2006).

Studies investigating compensation for an acute respiratory acidosis in fishes have been conducted on a relatively limited number of species including a few elasmobranchs [e.g. big skate *Raja ocellata* (Wood et al., 1990), dogfish *Scyliorhinus stellaris* (Heisler et al., 1988), starspotted dogfish *Mustelus manazo* (Hayashi et al., 2004)] or several teleosts [e.g. rainbow trout *Oncorhynchus mykiss* (Larsen and Jensen, 1997; Wood and LeMoigne, 1991), carp *Cyprinus carpio* (Claiborne and Heisler, 1984), European eel *Anguilla anguilla* (McKenzie et al., 2002), Conger eel *Conger conger* (Toews et al.,

1983), brown bullhead *Ictalurus nebulosus* (Goss et al., 1992), Japanese founder *Paralichthys olivaceus* (Hayashi et al., 2004), and yellowtail *Seriola quinqueradiata* (Hayashi et al., 2004)]. In general, when these fishes experience an increase in blood PCO_2, there is a corresponding rapid reduction in pH_e, which is then compensated over the following 24-96 h. The degree of pH_e reduction depends on the severity of acidosis and the buffer capacity of the blood. Bicarbonate and non-bicarbonate buffers help minimize the magnitude of pH disturbance, with the former being the CO_2-HCO_3^- system and the latter including phosphate buffers and haemoglobin (Hb) (due to the presence of histidine and their associated imidazole side chains that buffer H^+ at physiological pH) (Shartau and Brauner, 2014). Fishes, in general, have lower blood and tissue buffer values than other vertebrates (Cameron, 1989a; Heisler, 1984). Within the blood, however, buffer values vary among fishes, with the more basal groups (chondrichthyans, basal actinopterygians) having higher blood buffer values than teleosts (Berenbrink et al., 2005). When the capacity of the blood to buffer against acid-base disturbances is exceeded, pH changes occur and compensation typically occurs by net transport of acid-base equivalents between the fish and environment, with the gills, kidney and intestine all involved; the gills are believed to account for >90% of the net acid-base relevant ion transport during pH compensation (Brauner and Baker, 2009; Heisler, 1984).

In a few fish species, models of the cellular mechanism(s) underlying compensation of the extracellular compartment in response to an acidosis have been developed. Within the gill epithelium of *O. mykiss*, mitochondrion rich cells (MRCs) (or ionocytes) are believed to be the primary site of extracellular acid-base regulation. Two populations of MRCs exist, those with peanut lectin agglutinin (PNA) binding sites on their apical membranes (PNA^+ MRCs) and those lacking such sites (PNA^- MRCs). PNA^- MRC are proposed to be responsible for acid excretion where it is believed that H^+ elimination occurs via an apical NHE, or a VHA coupled to an apical epithelial Na^+ channel (ENaC). The result is hyperpolarization of the plasma membrane by transporting H^+ via VHA across the membrane, resulting in a favorable electrochemical gradient for diffusion of Na^+ via ENaC (Hwang et al., 2011). Net acid excretion is then achieved by the combined actions of apical H^+ efflux and basolateral HCO_3^- influx. Exchange of HCO_3^- is believed to occur via a HCO_3^-/Cl^- exchanger, such as those found in the SLC4

or SLC26 family and by the Na^+/HCO_3^- co-transporter (NBC – also found in the SLC4 family) (Evans et al., 2005; Hwang et al., 2011; Parks et al., 2009; Perry et al., 2009). The PNA^+ MRC is proposed to be responsible for base excretion in which the apical membrane HCO_3^-/Cl^- exchanger links Cl^- uptake to HCO_3^- excretion. Apical membrane HCO_3^- efflux along with basolateral H^+ efflux, via a VHA, would result in net transepithelial base excretion (Gilmour and Perry, 2009; Hwang et al., 2011). Using these membrane transporters, net acid-base equivalents can be transported from the blood to the environment to ensure pH homeostasis. In other freshwater fishes, the proposed mechanisms are similar to *O. mykiss*; for example, freshwater zebrafish *Danio rerio* (Gilmour and Perry, 2009), tilapia *Oreochromis mossambicus* (Hwang et al., 2011) and medaka *Oryzias latipes* (Hsu et al., 2014) use an apical NHE to remove H^+ catalyzed from CO_2, while HCO_3^- is moved to the blood via basolateral Na^+/HCO_3^- (NBC) or Cl^-/HCO_3^- (AE).

Regardless of the specific cellular mechanism(s) employed, in most fishes studied to date, compensation of pH_e during hypercarbia exposure is associated with a net increase in plasma $[HCO_3^-]$ that is matched by an equimolar reduction in plasma $[Cl^-]$ (Brauner and Baker, 2009; Heisler, 1984). The extent of this HCO_3^- elevation, however, appears to be limited, in that plasma $[HCO_3^-]$ rarely exceeds 27–33 mM during exposure to acute hypercapnia, which is referred to as the "apparent bicarbonate concentration threshold" (Heisler, 1984). This threshold is associated with an absence of complete pH_e compensation in most fishes during acute exposure to CO_2 tensions beyond 2–2.5 kPa PCO_2 (Baker et al., 2009a; Brauner and Baker, 2009) (Fig. 1.3). Although the basis of this threshold is unknown, recent work has supported the hypothesis that pH_e compensation during acute hypercarbia may be limited by the relative decrease in plasma Cl^- levels to avoid hypochloremia (Baker et al., 2015). Teleosts typically have plasma $[Cl^-]$ of 125 – 168 mM (Edwards and Marshall, 2013), of which approximately 17-20% can be exchanged with HCO_3^- before the bicarbonate concentration threshold is reached at approximately 27-33 mM HCO_3^-. In fish with higher plasma $[Cl^-]$, a similar pattern is observed; for example, the osmo- and iono-conforming Pacific hagfish *Eptatretus stoutii* has a plasma $[Cl^-]$ of ~458 mM and, perhaps as a result, hagfish are able to increase plasma $[HCO_3^-]$ to >80 mM, driving pH_e and pH_i recovery during exposure to severe

hypercarbia (PCO_2 of ~6.5 kPa) (Baker et al., 2015). Compensation of pH_e during acute hypercarbia is affected by the physicochemical characteristics of the surrounding water, such as the levels of acid–base relevant counter-ions (Larsen and Jensen, 1997). In contrast to acute hypercarbia, chronic CO_2 exposure allows some teleosts to elevate $[HCO_3^-]$ well beyond this threshold, aiding pH_e compensation. *O. mykiss* subjected to increasing hypercarbia over three days to reach 3.5 kPa PCO_2, and maintained at this level for an additional three days, had a blood $[HCO_3^-]$ of 66 mM (Dimberg, 1988). Similarly, *A. anguilla* gradually exposed to and maintained at 6 kPa PCO_2 for six weeks had plasma $[HCO_3^-]$ of 73 mM (McKenzie et al., 2003). Differences between acute and chronic compensation indicate that different mechanisms may underlie compensation to long-term hypercarbia exposures, a possibility that remains relatively unexplored.

1.1.2 Intracellular compartment

Although all cells have the capacity for some degree of pH_i regulation (Boron, 2004; Occhipinti and Boron, 2015; Putnam and Roos, 1997; Vaughan-Jones et al., 2009), in animals that employ coupled pH regulation, cells cannot fully compensate pH_i during a large sustained reduction in pH_e; this has been thoroughly examined *in vivo* and *in vitro* in a number of species. In fishes that exhibit coupled pH regulation, compensation of pH_e and pH_i occurs over the initial 24–96 h during sustained hypercarbia exposure (Fig. 1.1), with pH_i compensation usually occurring more rapidly that of pH_e, partly because intracellular fluids typically display a lower pH than the extracellular blood environment, which places the pK' of the CO_2–HCO_3^- reaction (pK' = 6.1) closer to pH_i (typically 6.3 – 7.0). Thus, relatively less HCO_3^- is required to compensate pH_i compared to pH_e. This recovery is further aided by the greater buffering capacity of intracellular fluids, which moderates the initial pH disturbance (Brauner et al., 2004; Occhipinti and Boron, 2015; Ultsch, 1996).

1.1.3 In vivo studies of pH_i regulation

Findings from *in vivo* studies conducted on a relatively small selection of fishes, amphibians, reptiles and mammals have established that pH_i regulation is coupled to pH_e regulation. In fishes, respiratory and metabolic acidoses typically lead to reductions in

pH_i and pH_e. For example, *E. stoutii* exposed to hypercarbia exhibited reduced pH_e and pH_i of heart, brain, liver and muscle (Baker et al., 2015); *R. ocellata* exposed to hypercarbia had reduced pH_e and pH_i of heart, brain and muscle; lemon sole *Parophrys vetulus* exposed to hypercarbia had reduced pH_e and pH_i heart, brain and muscle (Wright et al., 1988), cod *Gadus morhua* exposed to hypercarbia had reduced pH_e and pH_i of heart, liver and muscle (Larsen et al., 1997), and *O. mykiss* experiencing hypercapnia had reduced pH_e and pH_i of brain and muscle (Wood and LeMoigne, 1991) (see Shartau et al., 2016a for an overview). Similarly, metabolic acidoses also reduced pH_i of liver and muscle in starry flounder *Platichthys stellatus* (Milligan and Wood, 1987a; Milligan and Wood, 1987b), and heart and muscle pH_i in sea raven *Hemitripterus americanus* (Milligan and Farrell, 1986).

In adult tetrapods, coupled pH regulation is observed in all taxa where pH_e and pH_i have been measured during an acute respiratory acidosis. Exposure of the cane toad *Bufo marinus* (Snyder and Nestler, 1991; Toews and Heisler, 1982), knight anole *Anolis equestris* and desert iguana *Dipsosaurus dorsalis* (Snyder et al., 1995) to 5 kPa PCO_2 for 1 h resulted in a respiratory acidosis with severe reductions in pH_e and pH_i; reductions in pH_e and pH_i were observed in western painted turtles *Chrysemys picta bellii* up to 6 h during a severe acute respiratory acidosis associated with diving (Wasser et al., 1991). Similarly, *Rana catesbeiana* tadpoles exposed to 5 kPa PCO_2 resulted in reduced pH_e and pH_i of tail muscle and liver (Busk et al., 1997). Simultaneous reductions in pH_e and pH_i also occur during an acute metabolic acidosis following exhaustive exercise in the salt-water crocodile *Crocodylus porosus* (Baldwin et al., 1995).

In mammals subjected to an acute respiratory acidosis, similar responses are observed. In adult dogs (Arieff et al., 1976) and cats (Yaksh and Anderson, 1987) pH_e and pH_i were reduced following exposure to ≥8 kPa PCO_2 for 3 h and 10 min, respectively. In guinea pigs exposed to 15 kPa PCO_2 there was an uncompensated reduction in pH_e and pH_i of lung, kidney, heart and muscle between 2 and 8 h of exposure, but at 7 days, pH_e and pH_i exhibited compensation of 68% and 80–106%, respectively; a response indicative of coupled pH regulation (Wood and Schaefer, 1978). This pattern has been corroborated in rats (Gonzalez and Clancy, 1986b) and hamsters (Malan et al., 1985) during acute hypercarbia exposure. Thus, in all adult amniotes

investigated to date *in vivo* (dog, cat, rat, hamster, guinea pig, western painted turtle, knight anole and desert iguana), an acute respiratory acidosis results in reduced pH_e and pH_i, and compensation occurs through coupled pH regulation, as has been the case for a relatively small number of fish species that have been examined.

Although vertebrates are the focus of this thesis, it is worth noting that limited studies on invertebrates subjected to respiratory acidoses demonstrate coupled reductions in pH_e and pH_i, similar to that of vertebrates. In the few studies where both pH_e and pH_i have been measured during acute severe hypercarbia, reductions in pH_e and pH_i in a land snail *Otala lactea* (Barnhart and McMahon, 1988), deep sea bivalve *Acesta excavata* (Hammer et al., 2011), cuttlefish *Sepia officinalis* (Gutowska et al., 2010) and peanut worm *Sipunculus nudus* (Portner et al., 1998) have been observed.

1.1.4 In vitro pH_i regulation

In the many cell culture studies that have examined pH_i regulation following transitory reductions in pH_e (Bouyer et al., 2004; Filosa et al., 2002; Furimsky et al., 1999; Goldstein et al., 2000; Huynh et al., 2011b; Liu et al., 1990; Nottingham et al., 2001; Ritucci, 2005; Salameh et al., 2014), only a few have been conducted in the presence of sustained and elevated CO_2. For example, isolated trout hepatocytes were unable to recover pH_i in the presence of hypercarbia (Huynh et al., 2011b); this dependency of pH_i on pH_e is consistent with previous studies in trout and carp during chemically-induced anoxia (Krumschnabel et al., 2001).

Other studies using metabolic acid–base challenges are informative about the relationship between pH_i and pH_e. The general pattern shown in these studies in vertebrates is that (1) reducing pH_e causes pH_i to be reduced and (2) complete recovery does not occur until the starting pH_e is re-established (Occhipinti and Boron, 2015; Putnam and Roos, 1997; Vaughan-Jones et al., 2009). For example, when a metabolic acidosis was induced in a variety of mouse cell types (hippocampal neurons, astrocytes, medullary raphe neurons, colon cancer cells, skeletal muscle cells, macrophages, dendritic cells, melanocytes and keratinocytes) by lowering the external fluid [HCO_3^-] to reduce pH_e, the pH_i of all cell types was reduced and only fully recovered following the return of pH_e to control values (Salameh et al., 2014). Generally, *in vitro* studies indicate

that acute changes in external or environmental pH will rapidly affect pH_i. Although pH_i recovers in all cells once the source of the external acidosis is removed, most vertebrate cells are unable to avoid an acute reduction in pH_i when pH_e is reduced in a cell culture environment, characteristic of coupled pH regulation (Fig. 1.1).

This relationship between pH_e and pH_i is also observed during early development. When mouse (Siyanov and Baltz, 2013; Zhao and Baltz, 1996), hamster (Lane, 1999), bovine (Lane and Bavister, 1999) and human (Phillips et al., 2000) preimplantation embryos are subjected to an acidosis or alkalosis, they exhibit a decrease or increase in pH_i, respectively. These embryonic cells can typically compensate pH_i once environmental pH is returned to control values, although mammalian oocytes may not possess the capacity for pH_i regulation initially, instead relying on surrounding granulosa cells to correct ooplasmic pH (FitzHarris and Baltz, 2009). However, in sea urchins, larvae are able to fully compensate pH_i of primary mesenchyme cells during a hypercarbic-induced acidosis, and are able to accomplish this in the absence of pH_e compensation in the body cavity (Stumpp et al., 2012).

1.1.5 Cellular mechanisms of pH_i regulation

The above studies indicate that pH_i compensation during acid-base disturbances is almost always associated with pH_e compensation and thus pH_i is dependent on some degree of extracellular control of pH. Due to the importance of pH_i regulation, numerous studies have investigated the transporters involved in a variety of cell types in various species. The transporters involved in pH_i regulation generally include isoforms of acid-transporting Na^+/H^+ exchanger (NHE), V-type H^+-ATPase (VHA), and base-transporting HCO_3^-/Cl^- [anion exchangers (AE) also referred to as Cl^-/HCO_3^- exchanger (CBE)] and Na^+/HCO_3^- co-transporter (NBC) families. In general, during an acidosis, cells extrude H^+ to the extracellular space via extrusion of intracellular H^+ or uptake of extracellular HCO_3^-; the net effect being an increase in pH_i. The response to an alkalosis is the opposite, with the cell seeking to increase net H^+ concentration. In addition to those classic acid-base transporters, a lactate-H^+ cotransporter (monocarboxylate transporter; MCT) has been found to function during hypoxia (where metabolic acidosis typically occurs) to remove intracellular lactate and H^+. The transport of H^+ via MCT is facilitated

by carbonic anhydrase (CA) which catalyzes the $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ reaction to provide the MCT with H^+ (Parks and Pouysségur, 2017; Vaughan-Jones et al., 2009).

These mechanisms have been investigated in various vertebrate cell culture studies, including teleost hepatocytes (Ahmed, 2006; Furimsky et al., 2000; Huynh et al., 2011b), mammalian preimplantation embryos (FitzHarris and Baltz, 2009; Siyanov and Baltz, 2013), cardiac cells in avian embryos (Liu et al., 1990) and mammalian cardiac cells (Vaughan-Jones et al., 2009). Additionally, owing to the putative role of pH changes in cancer tumors, there is interest in understanding the mechanism of pH regulation in tumor cells. These cells are very resistant to chronic external acidosis, which is made possible via efficient pH regulatory systems. The tumor environment can reach nearly pH 6, yet tumor cells commonly demonstrate an alkaline pH_i despite this chronic metabolic acidosis, which is favorable for cellular metabolism and proliferation (Parks and Pouysségur, 2017; Reshkin et al., 2014); pH_i regulation in tumor cells may represent the extreme capacity for pH_i regulation in vertebrates, despite their maladaptive nature to the organism.

1.1.6 pH_e and pH_i regulation beyond the bicarbonate concentration threshold

The above studies indicate there is an intimate relationship between the regulation of pH_e and pH_i, and the inability to regulate the former limits the regulation of the latter. While vertebrates may tolerate extracellular acidoses of prolonged periods, they are not typically tolerant of severe changes in pH_i, even if pH_e recovers. In marine fishes, intracellular acidosis of heart was suggested to be the cause of mortality even though pH_e was compensated over the following 8 h in response to severe hypercarbia (Hayashi et al., 2004); similarly, reduction in muscle pH_i, not pH_e, was hypothesized to be the ultimate cause of mortality in trout following exhaustive exercise (Wood et al., 1983).

In fishes, the dependence of pH_i regulation on pH_e is particularly problematic when considering the limit of pH_e compensation during CO_2 exposure imposed by the bicarbonate concentration threshold (Fig. 1.3). As previously indicated, typically fishes cannot compensate pH_e beyond *ca*. 2 kPa PCO_2, thus regulation of pH_i is also hindered in these fishes and represents a major limitation of coupled pH_e/pH_i regulation. Despite the putative limits restricting pH_e, and thus pH_i, compensation to approximately 2 kPa PCO_2,

many fishes appear to tolerate these conditions without the expected morbidity or mortality. Acid-base regulation in a few fishes exposed to >4 kPa $P\mathrm{CO_2}$ has revealed the use of a novel strategy of acid-base regulation in which $\mathrm{pH_e}$ is reduced, and remains uncompensated, but $\mathrm{pH_i}$ is remains tightly regulated (Baker et al., 2009a; Brauner et al., 2004; Heisler, 1982); this strategy is referred to as preferential $\mathrm{pH_i}$ regulation and is discussed below.

1.2 Preferential $\mathrm{pH_i}$ regulation

Preferential $\mathrm{pH_i}$ regulation is defined as $\Delta\mathrm{pH_i}/\Delta\mathrm{pH_e} \leq 0$ immediately following onset of an acid-base disturbance. This is associated with complete $\mathrm{pH_i}$ regulation of heart, brain, liver and muscle despite large reductions in $\mathrm{pH_e}$ that may approach 1 pH unit, and has been proposed to confer exceptional hypercarbia tolerance (Brauner and Baker, 2009; Shartau et al., 2016a). Preferential $\mathrm{pH_i}$ regulation was first documented during forced air breathing (induced by aquatic hypoxia) of *Synbranchus marmoratus*, the marbled swamp eel, an Amazonian air breathing teleost. The treatment resulted in an increase in blood $P\mathrm{CO_2}$ to 3.5 kPa after 96 h causing a reduction in $\mathrm{pH_e}$ but no change in heart or muscle $\mathrm{pH_i}$ (Heisler, 1982). Two decades following Heisler's work, Brauner et al. (2004) observed limited $\mathrm{pH_e}$ compensation but a remarkable ability for regulation of $\mathrm{pH_i}$ during short-term environmental hypercarbia in *Pterygoplichthys pardalis* armoured catfish, a tropical air breather found in the Amazon River. *Pterygoplichthys pardalis* preferentially regulated $\mathrm{pH_i}$ of heart, liver and white muscle during 24 and 72 h exposure to 1.9 and 4.3 kPa $P\mathrm{CO_2}$, respectively; during these exposures, $\mathrm{pH_e}$ was reduced and was not compensated for. The strategy of preferential $\mathrm{pH_i}$ regulation was considered to be rare amongst vertebrates by Brauner et al. (2004) and possibly associated with air breathing or living in ion-poor waters, such as those of the Amazon River basin.

More recently, Baker et al. (2009a) observed the first example of preferential $\mathrm{pH_i}$ regulation in a non-air breathing fish, *Acipenser transmontanus*, the white sturgeon. This is a basal euteleostom fish, which tightly protected $\mathrm{pH_i}$ despite severe $\mathrm{pH_e}$ reduction of *ca*. 0.8 pH units during 48 h exposure to 6 kPa $P\mathrm{CO_2}$. Similar to *S. marmoratus* and *P. pardalis*, *A. transmontanus* experience a large uncompensated reduction in $\mathrm{pH_e}$ during

hypercarbia exposure but fully protect pH of heart, brain, liver and white muscle. pH_i is exceptionally well protected, such that pH_i of heart and brain experience an increase in pH, becoming slightly alkalotic relative to their normocarbic pH (Baker et al., 2009a); exposure to 12 kPa PCO_2 for 6 h reduced pH_e by ~1.0 pH unit, while liver pH_i increased by 0.2 pH units (Baker and Brauner, 2012). Protection of pH_i during exposure to hypercarbia in *A. transmontanus* appears to be nearly instantaneous. When heart pH_i was measured in real time at 2-minute intervals using magnetic resonance imaging (MRI), there was no evidence for heart muscle pH_i ever decreasing (Baker, 2010). This use of preferential pH_i regulation during severe acute hypercarbia does not appear to be metabolically costly as whole animal metabolic rate during exposure to 6 and 12 kPa PCO_2 corresponded to 30 and 60% reduction in MO_2, respectively (Baker and Brauner, 2012).

That preferential pH_i regulation appears to confer exceptional CO_2 tolerance may be due to the protection of intracellular pH of critical tissues (e.g. heart and brain). While some marine fishes demonstrate tremendous capacity for pH_e compensation during severe acute hypercarbia exposure, mortality typically occurs despite recovery of pH_e; this is postulated to be due to changes to cardiac performance (e.g. cardiac output, contractility) which leads to a reduction in organismal oxygen supply (Hayashi et al., 2004). In contrast, cardiac performance is also fully protected during exposures up to 5 and 6 kPa PCO_2 in *P. pardalis* (Hanson et al., 2009) and *A. transmontanus* (Baker et al., 2011), respectively, which is believed to be associated with complete protection of heart pH_i. Reduction in brain pH is associated with loss of equilibrium in *Cyprinus carpio* (common carp) (Yoshikawa et al., 1994) and reduced muscle pH was hypothesized to be the cause of post-exercise mortality in *O. mykiss* (Wood et al., 1983).

1.2.1 Mechanisms of preferential pH_i regulation

The mechanism(s) of preferential pH_i regulation are unknown (Brauner and Baker, 2009), but likely involve transporters identified for pH_i regulation; however, the specific mechanism(s) could be most effectively be investigated using cell culture. *Acipenser transmontanus* primary liver cells were exposed to 6 kPa PCO_2 for 19-50 h experienced an initial pH_i reduction which was compensated despite a sustained

extracellular acidosis (Huynh et al., 2011a); this is in contrast to a similar study using trout hepatocytes exposed to hypercarbia as these cells never recovered pH_i while hypercarbia was maintained (Huynh et al., 2011b). The response *in vitro* differs from *in vivo* as liver pH_i in sturgeon is not reduced during hypercarbia (Baker and Brauner, 2012; Baker et al., 2009a), which suggests that liver pH_i regulation during hypercarbia is influenced by extrinsic factors (Huynh et al., 2011a). While preferential pH_i regulation is due to active transport of acid-base equivalents (Baker et al., 2009a), the relatively high buffer capacity of the intracellular compartments compared to the blood and extracellular fluid is likely beneficial in ensuring there are no initial pH_i changes at the onset of the acid-base disturbance.

1.3 Hypercarbia and acid-base regulation – a role for preferential pH_i regulation?

Due to the prevalence of hypercarbia in various environments, preferential pH_i regulation may be important for conferring CO_2 tolerance in these habitats. High environmental CO_2 is common in many environments, particularly in aquatic ecosystems (Marcé et al., 2015; McNeil and Sasse, 2016; Raymond et al., 2013; Reum et al., 2014), and may arise due to a number of factors, including high aquatic biomass, thermo-stratification and poor water mixing, surface vegetation, and anaerobic metabolism of microorganisms (Brauner and Baker, 2009; Ultsch, 1996). Since fishes comprise over half of all vertebrate species (~32,000) (Nelson, 2006), hypercarbia may be an important abiotic variable affecting life history and evolution of fishes (Hasler et al., 2016; Ultsch, 1987). Hypercarbia in tropical freshwater may reach >8% PCO_2 (Furch and Junk, 1997; Heisler, 1984; Li et al., 2013) and even temperate waters can experience naturally elevated PCO_2 (Atilla et al., 2011; Butman and Raymond, 2011; Weyhenmeyer et al., 2012); for example, the lower Columbia River can reach 870 μatm (*ca*. 0.9 kPa) PCO_2 (Park et al., 1969). These values are far in excess of current atmospheric CO_2 levels [400 μatm (*ca*. 0.04 kPa)] and still much greater than projected end of century CO_2 increases that have been predicted due to climate change [~1000 μatm (*ca*. 0.1 kPa)] (McNeil and Sasse, 2016).

Although coupled pH_e/pH_i regulation may be generally limited to PCO_2 <2 kPa, many fishes inhabit environments that may experience hypercarbia in excess of >8 kPa PCO_2 (Furch and Junk, 1997; Heisler, 1984), which is beyond their ability to compensate pH_e. These hypercarbic-prone environments contain a disproportionate percentage of the world's freshwater fishes, particularly within the Amazon and Mekong river basins, as 56% of watersheds with high fish species diversity are located in the tropics (Val et al., 2005). In the marine environment, there are natural CO_2 seeps at some locations which create localized hypercarbia that may reach as high as 7 kPa PCO_2 (Basso et al., 2015; Melzner et al., 2009).

In addition to aquatic hypercarbia, some terrestrial environments experience dramatically higher PCO_2 than ambient atmospheric levels. Some burrows of subterranean rodents may have PCO_2 ranging from 0.22-6.1 kPa (Burda et al., 2007; Shams et al., 2005). Typically, burrow gas composition remains constant, thus hypercarbia is chronic, however, depending on the soil type, some burrows may experience large changes in PCO_2 over the course of hours to days, and thus expose rodents to severe acute hypercarbia; under laboratory conditions, subterranean mole rat *Spalax sp*. can survive 15 kPa PCO_2 and 3 kPa PO_2 for at least 8 h without physiological or behavioural changes (Shams et al., 2005). In cave environments, PCO_2 may reach 6 kPa; in one cave system, numerous species inhabit high CO_2 regions, including troglobites and bats (Howarth and Stone, 1990). Nests of birds and reptiles naturally experience changes in CO_2 levels due to biotic (i.e. metabolic activity of embryos and microorganisms) and abiotic (i.e. diffusion gradients/barriers and nest composition) factors, often resulting in hypercarbic rearing environments for embryos; consequently, nest CO_2 can reach 5-8 kPa PCO_2 (Booth, 1998; Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984; Seymour et al., 1986).

The acid-base response in terrestrial animals exposed to these severely hypercarbic conditions has not been well investigated. While animals exposed to chronic hypercarbia in caves and burrows likely adjusted their blood-gas composition to account for those differences, it is not known what happens during acute exposure. Amongst embryonic amniotes only the extracellular response of chicken embryos to severe acute hypercarbia has been investigated (Everaert et al., 2011). Those studies demonstrate that

chicken embryos exposed to severe acute hypercarbia experience a typical respiratory acidosis, but there is minimal pH_e compensation when hypercarbia is maintained for up to 24 h (Andrewartha et al., 2014; Burggren et al., 2012); it is unknown how pH_i responds.

Due to the severe effects of hypercarbia, and especially given the limitations of coupled pH regulation, preferential pH_i regulation may play an important role as an acid-base regulatory strategy in a number of these species that regularly enter and experience hypercarbic conditions. At the start of this dissertation only four species were known to use preferential pH_i regulation (Fig. 1.4) but it remains uncertain how other species tolerate the severe acid-base disturbances they presumably experience during those conditions.

1.4 Preferential pH_i regulation: a basal euteleostom strategy or embryonic strategy?

Preferential pH_i regulation has been previously hypothesized to (1) confer exceptional CO_2 tolerance and (2) that it evolved in the basal actinopterygians, as it has not been observed in hagfish or elasmobranchs (Brauner and Baker, 2009). However, direct (*Siren lacertina* greater siren (Heisler et al., 1982)) and indirect (*Lepidosiren paradoxa* South American lungfish (Sanchez et al., 2005)) evidence for preferential pH_i regulation amongst the sarcopterygii suggests that it may have been used in basal Euteleostomi (Sarcopterygii + Actinopterygii) as a strategy of acid-base regulation in adults. Loss of preferential pH_i regulation may have occurred due to changes in physiology requiring coupled pH regulation and where environmental conditions were favorable to permit pH_e compensation. In teleosts, for example, the evolution of the Root effect likely necessitated regulation of the extracellular compartment during acidoses. This is because of the pH-sensitivity of Root effect hemoglobins, which exhibit a reduction in Hb-O_2 affinity as pH is reduced. Although hemoglobin is confined to the red cells, and teleosts possessing the Root effect regulate RBC pH over the short-term via beta-adrenergic Na^+/H^+ exchanger (βNHE) to safeguard O_2 transport, this mechanism

remains dependent on eventual pH_e recovery (Berenbrink et al., 2005; Shartau and Brauner, 2014). As the species where preferential pH_i regulation has been identified do not possess a Root effect, nor do they have RBC βNHEs (Berenbrink et al., 2005), RBC pH_i is not regulated (Baker et al., 2009a; Brauner et al., 2004) and thus H^+ exchange is largely passive across the RBC membrane. However, because Hb-O_2 affinity is not affected to a large degree by changes in pH in these species, an uncompensated pH_e acidosis may not be detrimental to O_2 transport. Based on these data, preferential pH_i regulation was hypothesized to have evolved in the basal euteleostomi and retention or loss in extant euteleostomi groups was driven by either environmental (e.g. hypercarbia) and/or physiological constraints (e.g. Root effect).

Alternatively, preferential pH_i regulation in adults may represent the retention of the embryonic capacity for intracellular pH regulation. Although few studies have characterized pH_i regulation during vertebrate development, there is evidence that early-stage embryos are capable of pH regulation just after fertilization, and mammalian oocytes and embryos are able to recover from an intracellular acid-base disturbance of almost 1 pH unit (FitzHarris and Baltz, 2009; Lane, 1999). Work on fish has also shown this pattern as early-stage zebrafish embryos exposed to 3.3 kPa PCO_2 for 2 h *in vitro* display a respiratory acidosis, but are still able to restore pH_i to pre-hypercarbic values (Molich and Heisler, 2005). Similarly, sea urchin larvae are able to fully compensate pH_i of primary mesenchyme cells during a hypercarbic-induced acidosis in the absence of pH_e compensation in the body cavity (Stumpp et al., 2012), suggesting that preferential pH_i regulation may not be limited to vertebrates; no evidence presently exists for preferential pH_i regulation in adult invertebrates (Shartau et al., 2016a). These studies indicate that during an acute acidosis cells have the capacity for pH_i compensation at the earliest developmental time points. It is unknown for how long embryos retain this capacity for pH_i regulation, as it may be reduced or enhanced following the appearance of the extracellular space and the growth of organs involved in regulating pH_e; additionally, the above findings are from *in vitro* studies and, as indicated previously, these may or may not be representative of *in vivo* responses during an acid–base disturbance. Beyond the earliest developmental stages, acid–base regulation has been poorly studied in embryonic vertebrates; however, several studies to date have examined the response of

late-stage chicken (*Gallus gallus*) embryos to respiratory and metabolic acidoses (Everaert et al., 2011). Exposing chicken embryos *in vivo* to severe acute respiratory or metabolic acidosis for up to 24 h results in large reductions in pH_e that are not fully compensated yet the embryos survive (Burggren et al., 2012; Mueller et al., 2014); while pH_i was not measured, the changes in pH_e are consistent with pH_e changes observed in fish that preferentially regulate pH_i (Brauner and Baker, 2009). Adult amniotes use coupled pH regulation (Cameron, 1989b; Shartau et al., 2016a), but during embryonic development in which high CO_2 exposure may occur, they may utilize preferential pH_i regulation to cope with the acid-base disturbance. Based on this limited embryonic data, I hypothesized that preferential pH_i regulation is an embryonic strategy of acid-base regulation that is retained or lost throughout development. Insufficient information existed to support or reject these ideas pertaining to preferential pH_i regulation as either a basal euteleostom or embryonic strategy of acid-base regulation; this was focus of investigation for my thesis.

1.5 Thesis objective and organization

Preferential pH_i regulation may provide a way to avoid the putative limits of coupled pH_e/pH_i regulation, and thus allow those species to tolerate and survive challenging levels of CO_2 exposure. The pH_e and pH_i responses of species using preferential pH_i regulation is wholly different from those using coupled pH regulation (Brauner and Baker, 2009; Shartau et al., 2016a); however, little about preferential pH_i regulation is known. The objective of this thesis is to investigate the usage, distribution/prevalence, and origin of preferential pH_i regulation as a strategy of acid-base regulation in vertebrates. In this thesis, the general objective is addressed in the subsequent four chapters: 1) Is preferential pH_i regulation a general strategy of acid-base regulation in white sturgeon subjected to a range of acid-base disturbances? 2) Is preferential pH_i regulation a common strategy of acid-base regulation among a diverse range of fish species? 3 and 4) How does the strategy of acid-base regulation shift throughout development in amniotes known to use coupled pH regulation as an adult?

1.5.1 Is preferential pH_i regulation a general strategy of acid-base regulation in white sturgeon subjected to a range of acid-base disturbances?

Studies observing preferential pH_i regulation have all been conducted during hypercarbic conditions (Shartau et al., 2016a) and it is uncertain whether preferential pH_i regulation also confers protection against non-hypercarbic induced acidoses. In *P. pardalis* and *A. transmontanus*, it is clear that preferential pH_i regulation allows tissues to be fully protected against a range of hypercarbic exposures. However, fishes are often exposed to various other conditions that may pose challenges for acid-base regulation (e.g. hypoxia and exercise) and it is unknown if preferential pH_i regulating species are able to confer the same degree of protection during these other types of acid-base disturbances. As the origins of respiratory and metabolic acidoses are different, it is uncertain whether preferential pH_i regulation functions in the latter, or if it does, if it confers the same degree of pH_i protection; more specifically, is preferential pH_i regulation a general strategy of acid-base regulation?

Chapter 2 seeks to address this question using *A. transmontanu*s as their capacity for preferential pH_i regulation has been investigated numerous times during hypercarbia (Baker and Brauner, 2012; Baker et al., 2011; Baker et al., 2009a; Shaughnessy et al., 2015). I hypothesized that the tremendous capacity for preferential pH_i regulation in *A. transmontanus* during hypercarbia reflects the use of preferential pH_i regulation as a general strategy of acid-base regulation. This was investigated by subjecting *A. transmontanus* to conditions creating severe metabolic (exhaustive exercise, anoxia, and air exposure) and non-hypercarbic respiratory acidoses (hyperoxia). Following exposure to various treatments, fishes were sampled for pH_e and pH_i of heart, brain, liver, and white muscle to determine their acid-base regulatory response. This chapter will also inform on whether non-hypercarbic acidoses can be used in future studies to assess the presence or absence of preferential pH_i regulation, and if existing literature can be used to infer on the strategy of acid-base regulation.

1.5.2 Is preferential pH_i regulation a common strategy of acid-base regulation among a diverse range of fish species?

At the start of this dissertation research, only three fishes had been identified to use preferential pH_i regulation, suggesting limited use of preferential pH_i regulation in fishes (Fig. 1.4). However, as few studies have measured pH_e and pH_i concurrently, and only amongst a relatively limited number of species, it is uncertain as to whether preferential pH_i regulation is truly a rare strategy as suggested by Brauner et al. (2004). As many fishes inhabit environments prone to severe hypercarbia, with PCO_2 reaching well beyond the putative capacity for pH_e regulation (Brauner and Baker, 2009; Heisler, 1984), it is likely that preferential pH_i regulation is more widely used; especially given that two of the three species are both tropical air breathers, it seems likely that other tropical/Amazonian fishes would possess this ability for pH regulation. Aside from those three preferential pH_i-regulating fishes, the general strategy for acid-base regulation amongst fishes (and vertebrates) is coupled pH_e/pH_i regulation. But, is preferential pH_i regulation a unique strategy in a world characterized by coupled pH regulation, or is the former more common than current data suggest?

Chapter 3 seeks to address the question of whether preferential pH_i regulation is a common strategy of acid-base regulation in response to severe acute hypercarbia. I hypothesize that preferential pH_i regulation is a widespread strategy amongst fishes used during severe acute hypercarbia. This was investigated by conducting a survey that included 20 fish species from groups ranging from basal to derived, including lamprey, lungfish, elasmobranchs, basal actinopterygians, and various teleosts to assess the presence or absence of preferential pH_i regulation. Using a CO_2 tolerance assay developed for this purpose, the acute CO_2 tolerance of fishes was first determined, then presence or absence of preferential pH_i regulation was determined directly via pH_e/pH_i measurements following hypercarbia exposure, or indirectly via CO_2 tolerance. This chapter will provide a better understanding of the distribution of preferential pH_i regulation, and how fishes tolerate and survive in hypercarbic environments.

1.5.3 How does the strategy of acid-base regulation shift throughout development?

Acid-base regulation is a critical physiological process and regulation is present

early in development, starting with egg and zygote (FitzHarris and Baltz, 2009; Johnson and Epel, 1981; Lane, 1999; Molich and Heisler, 2005). While these early developmental stages do not appear to completely avoid pH_i changes when subjected to such conditions designed to induce an acid-base disturbance, they are capable of adjusting intracellular pH (Johnson and Epel, 1981; Molich and Heisler, 2005). Experiments at these early developmental stages demonstrate cells have robust capacity for pH_i regulation; however, early in development the tissues have not formed, nor is the extracellular space developed, which makes comparisons with adult acid-base strategies inherently difficult. Following the development of tissues and the extracellular space, there may be changes in acid-base regulatory physiology as well, however, this has not been investigated.

Embryonic invertebrate (Shartau et al., 2010), fish (Ciuhandu et al., 2007) and amniote (Booth, 1998; Grigg et al., 2010) species may experience challenging environmental conditions (e.g. hypoxia) that are more severe than they would experience as adults. These differences between embryo and adult may lead to different degrees of acid-base disturbances for which different strategies may be employed, such as preferential pH_i regulation or coupled pH regulation. Little is known about acid-base regulation during embryonic development in most animals; however, due in part to the large size of amniote embryos, some work has looked at bird and reptile embryos. Adult amniotes do not typically experience severe hypercarbia naturally, but when they are subjected to hypercarbia they utilize coupled pH regulation; this response in embryos has not been well investigated (Everaert et al., 2011), which is perhaps surprising given the severity of hypercarbia some embryonic reptiles and birds may experience (Booth, 1998; Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984; Seymour et al., 1986). Embryonic amniotes do not have the similar physiological tools of adults to respond to respiratory acidoses via adjustment to ventilation and they may be constrained by nest and eggshell diffusion (Erasmus et al., 1971), thus, their acid-base regulatory response may differ. Studies on embryonic chickens suggest they are resilient to very high CO_2 and can tolerate prolonged periods of an uncompensated reduction in pH_e, suggesting amniote embryos may have a greater capacity for pH_i protection than adults.

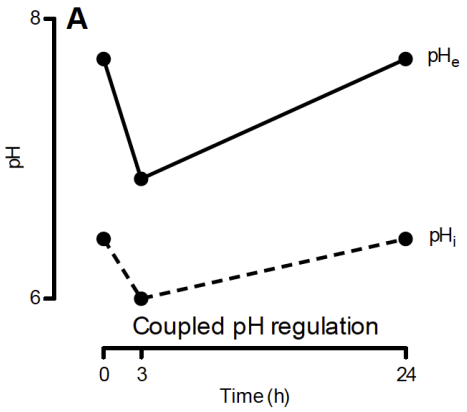
Chapter 4 investigates the strategy of acid-base regulation during development of a hypercarbic tolerant amniote, *Chelydra serpentina* (common snapping turtle); adult

turtles are known to use coupled pH regulation (Wasser et al., 1991). I hypothesized that embryonic *C. serpentina* would preferentially regulate pH_i in response to acid-base disturbances. This was investigated by exposing *C. serpentina* embryos at 70 and 90% to hatch, and yearlings to severe acute hypercarbic hypoxia exposure and measuring pH_e and pH_i of various tissues. This chapter provides the first insight into how amniote embryos regulate both pH_e and pH_i during severe acute acid-base disturbances, and it will inform on how acid-base regulation changes throughout development.

1.5.4 Is preferential pH_i regulation an embryonic strategy in amniote embryos?

Indirect evidence suggests chicken embryos use preferential pH_i regulation during severe acid-base challenges (Andrewartha et al., 2014; Burggren et al., 2012) and results from Chapter 4 indicates *C. serpentina* embryos preferentially regulate pH_i (Shartau et al., 2016b); thus other embryonic amniotes subjected to severe hypercarbia may employ a similar strategy. Another amniote that is known to experience and tolerate severe hypercarbia during development are crocodylians (Eme and Crossley, 2015), which lay eggs in nests where PCO_2 can reach 2-8 kPa (Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984). Little is known about acid-base regulation in oviparous amniote embryos (Everaert et al., 2011).

Chapter 5 investigates the strategy of acid-base regulation in another embryonic amniote. Preferential pH_i regulation is likely to be used by embryonic amniotes that are subjected to severe hypercarbic conditions in nest environments. I hypothesized that *Alligator mississippiensis* (American alligator) embryos preferentially regulate pH_i during severe acid-base disturbances. This was investigated by exposing *A. mississippiensis* embryos at 70% to hatch to severe hypercarbic hypoxia and measuring pH_e and pH_i; the same developmental time was used at which *C. serpentina* displayed the most robust pH_i regulation. This chapter provides insight into crocodylian acid-base regulation during development and, along with *C. serpentina*, contribute to understanding how pH regulation in amniotes, and thus, vertebrates.



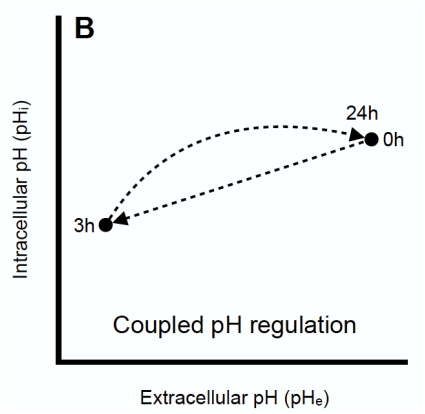


Figure 1.1: Representation of the typical response of vertebrates utilizing coupled pH regulation during acute sustained hypercapnia. In species utilizing coupled pH regulation, a hypercarbia-induced respiratory acidosis (initiated at t=0) leads to a rapid reduction in both pH_e and pH_i, with maximal pH depression occurring typically by 3 h or less. Recovery of pH then occurs by 24 h, but the rate of compensation depends on the severity of the pH depression and the ability for net acid excretion to the environment. pH_i is often compensated more rapidly than pH_e but complete pH_i compensation generally requires >50% of complete pH_e recovery.

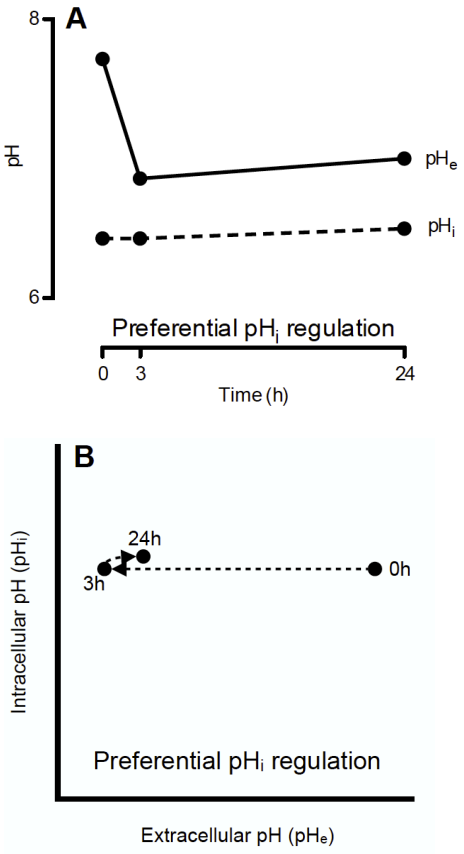


Figure 1.2: Representation of the typical response of vertebrates utilizing preferential intracellular pH (pH_i) regulation during acute sustained hypercapnia. In species utilizing preferential pH_i regulation, a hypercarbia-induced respiratory acidosis leads to a rapid reduction in pH_e but no change (or even a slight increase) in pH_i. During hypercarbia, pH_i remains independent of the sustained, uncompensated reduction of pH_e for periods >24 h.

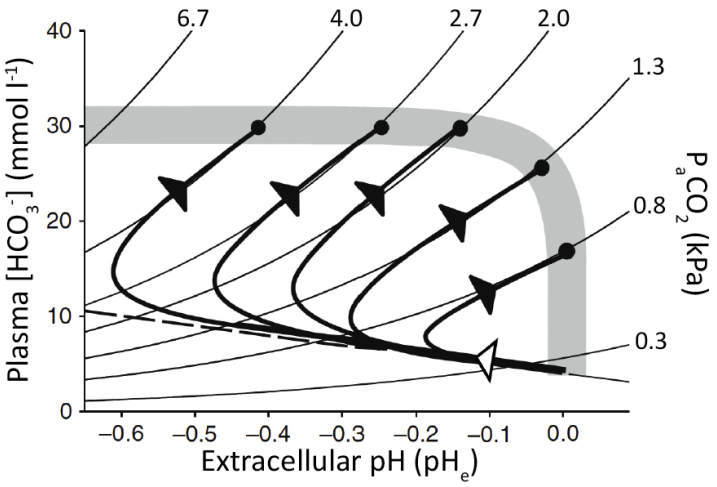


Figure 1.3: A theoretical representation of the typical extracellular pH (pH_e) response to short-term (<5 days) hypercarbia in fish. Transfer from normocarbia to hypercarbia results in extracellular pH (pH_e) falling along the blood non-bicarbonate buffer line, which is indicated by the black open arrowhead. pH_e then recovers along a given PCO_2 isopleth via a net increase in HCO_3^- in exchange for Cl^- as indicated by black filled arrowheads. Black filled circles represent final pH_e values that would be achieved based upon limits to net HCO_3^- accumulation within 24-96 h exposure to hypercarbia. Shaded bar indicates the greatest pH compensation able to occur at each CO_2 tension, which is believed to be limited by constraints on HCO_3^- accumulation and is termed the 'bicarbonate concentration threshold'. Most fishes studied to date cannot increase plasma HCO_3^- beyond 27-35 mmol l^{-1} (Baker et al., 2015; Brauner and Baker, 2009; Heisler, 1984). Consequently, compensation for an acute respiratory acidosis (<96 h) during exposure to hypercarbia is incomplete above a PCO_2 of *ca*. 2 kPa in most fishes. Figure modified from Brauner and Baker (2009).

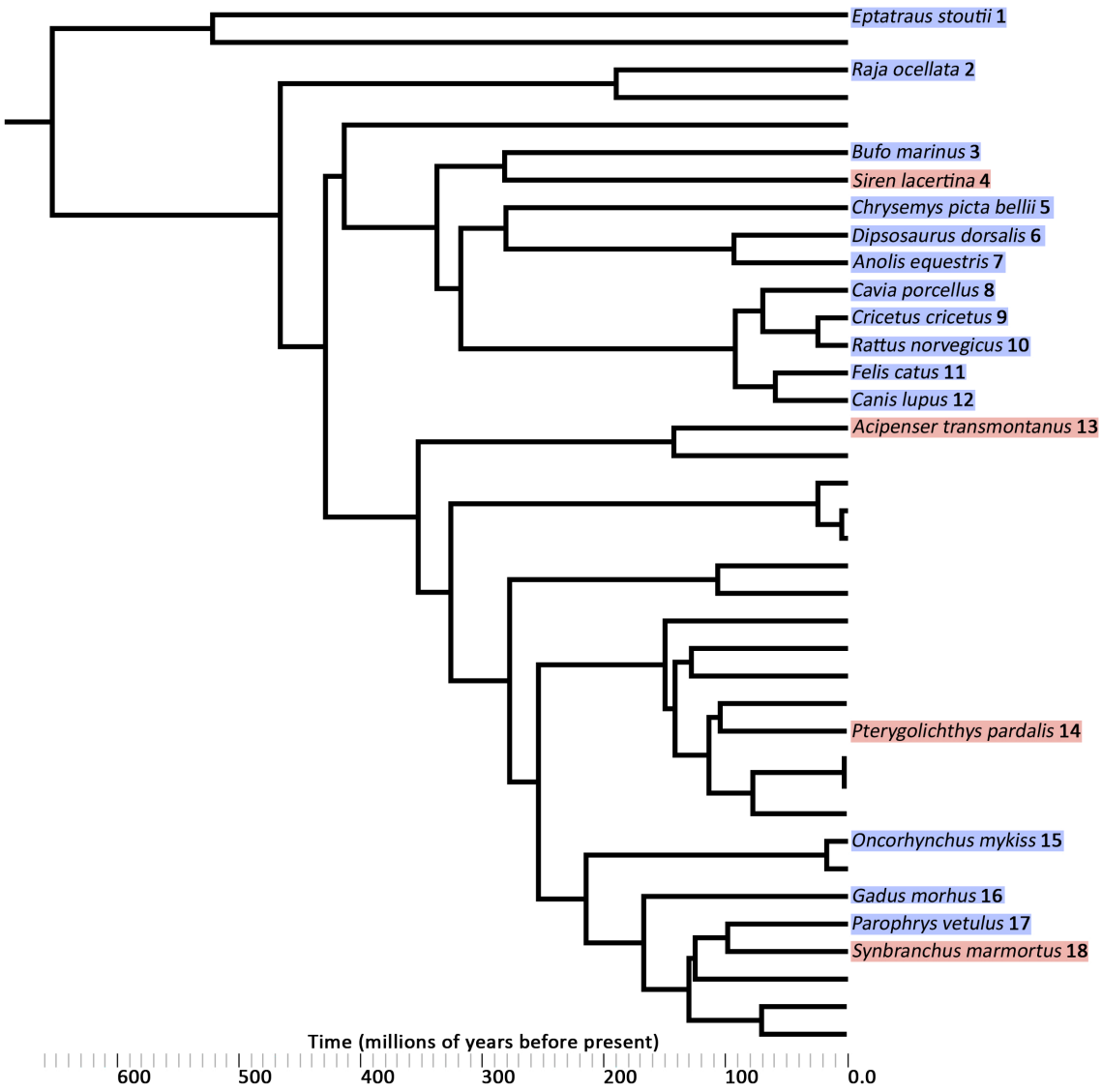


Figure 1.4: Phylogeny showing distribution of preferential intracellular pH (pH_i) regulation and coupled pH regulation amongst vertebrates when exposed to acute >2 kPa PCO_2 prior to dissertation research. Species using preferential pH_i regulation during severe acute hypercarbia are indicated in pink, while those using coupled pH regulation are indicated in blue. Empty branches indicate species that will be examined in this dissertation – see Fig. 6.1 for the filled in phylogeny. References are indicated by numbers following species name. 1(Baker et al., 2015), 2(Wood et al., 1990), 3(Snyder and Nestler, 1991), 4(Heisler et al., 1982), 5(Wasser et al., 1991), 6(Snyder et al., 1995), 7(Snyder et al., 1995), 8(Malan et al., 1985), 9(Wood and Schaefer, 1978), 10(Gonzalez and Clancy, 1986a), 11(Yaksh and Anderson, 1987), 12(Arieff et al., 1976), 13(Baker et al., 2009a), 14(Brauner et al., 2004), 15(Wood and LeMoigne, 1991), 16(Larsen et al., 1997), 17(Wright et al., 1988), 18(Heisler, 1982). Phylogenetic relationships are based on (2009) and branch lengths are taken

from various references utilizing fossil and molecular estimates of divergence times (Aschliman et al., 2012; Betancur-R et al., 2013; Betancur-R et al., 2015; Blair, 2005; Macqueen and Johnston, 2014; Meredith et al., 2011; Zhang et al., 2013); the phylogenetic tree was created using Mesquite (Maddison and Maddison, 2017).

Chapter 2: White Sturgeon (*Acipenser transmontanus*) Acid-Base Regulation Differs in Response to Different Types of Acidoses

2.1 Introduction

Acid-base regulation is one of the most important physiological processes in vertebrates due to the effect of pH on proteins, as changes in pH typically alter protein charge, which can change protein function, and ultimately reduce whole animal performance (e.g. reduce heart and skeletal muscle contractility, and alter metabolic pathways) (Occhipinti and Boron, 2015; Putnam and Roos, 1997). Challenges to acid-base homeostasis may have respiratory or metabolic origins, or a combination of the two. A respiratory acidosis results in a reduction in blood pH (extracellular pH; pH_e) and intracellular pH (pH_i) due to an increase in blood PCO_2. In fishes, compensation of pH_e primarily occurs at the gills via net exchange of acid-base relevant ions, whereby an increase in plasma [HCO_3^-] occurs and is generally associated with an equimolar decrease in plasma [Cl^-], which gradually compensates pH_e at an elevated blood PCO_2 (Brauner and Baker, 2009; Heisler, 1984; Perry and Gilmour, 2006; Shartau et al., 2016a). Similarly, metabolic acidoses reduce pH_e and pH_i, but the acidosis often originates from the cells as a consequence of increased H^+ and lactate production associated with anaerobic glycolysis (Robergs et al., 2004). Compensation of a metabolic acidosis is primarily dependent on net exchange of acid-base relevant ions at the gills (Evans et al., 2005; Hwang et al., 2011) and to a lesser degree, excretion of acidic equivalents in the form of titratable acidity and ammonium ions through renal pathways (Kwong et al., 2014). Typically, some degree of pH_e compensation is required for pH_i compensation as the two are coupled (referred to as 'coupled pH regulation', see Shartau et al., 2016a). Some fishes, such as the white sturgeon *Acipenser transmontanus*, however, do not follow this pattern and completely regulate pH_i, often at the expense of pH_e regulation

and in the face of severe maintained reductions in pH_e, termed preferential pH_i regulation (Brauner and Baker, 2009; Shartau et al., 2016a).

Acipenser transmontanus are basal actinopterygians, belonging to the Acipenseriformes and are found along the Pacific coast of North America in the Fraser, Columbia, Sacramento and San Joaquin river systems (Hildebrand et al., 2016). They are one of the most CO_2 tolerant fishes, able to tolerate a hypercarbic-induced respiratory acidosis of at least 12 kPa PCO_2 for 6 h, which reduces pH_e by nearly 1 pH unit and the extracellular acidosis remains uncompensated over this time period (Baker and Brauner, 2012). This ability is attributed to preferential pH_i regulation of heart, brain, liver and muscle during these exposures (Brauner and Baker, 2009; Shartau et al., 2016a); protection of muscle pH_i has been observed for up to 10 days at 6 kPa PCO_2 (Shaughnessy et al., 2015). Hypercarbia in *A. transmontanus* has been well studied (Baker and Brauner, 2012; Baker et al., 2011; Baker et al., 2009a; Cech and Crocker, 2002; Crocker and Cech, 1998; Shartau et al., 2017b), and while severe hypercarbia (> 3 kPa PCO_2) is unlikely to be widely encountered in natural settings, it may occur in aquaculture settings (Crocker and Cech, 1996). Mild hypercarbia (< 1 kPa PCO_2), however, may be frequently experienced by *A. transmontanus* in their environment and in hatchery settings (Crocker and Cech, 1998). Hypercarbia tolerance is believed to be attributed to the capacity for preferential pH_i regulation as other species (e.g. marbled swamp eel *Synbranchus marmoratus*, armoured catfish *Pterygoplichthys pardalis*, striped catfish *Pangasianodon hypophthalmus*, spotted gar *Lepisosteus oculatus* and alligator gar *Atractosteus spatula*) exhibiting this degree of hypercarbia tolerance also preferentially regulate pH_i (see Shartau and Brauner, 2014; Shartau et al., 2016a).

Recently, it was observed that preferential pH_i regulation confers protection against acid-base disturbances in addition to those induced by hypercarbia in the tropical air breathing *P. pardalis* (Harter et al., 2014). The authors concluded that preferential pH_i regulation may represent a general strategy of acid-base regulation in this species (Harter et al., 2014). Given the tremendous capacity *A. transmontanus* have for preferential pH_i regulation during hypercarbia, we hypothesized that *A. transmontanus*, similar to *P. pardalis*, utilize preferential pH_i regulation as a general strategy of acid-base regulation, irrespective of the origin of the disturbance.

To test this hypothesis, *A. transmontanus* were subjected to either respiratory or metabolic acidoses and their acid-base response was measured. Determining the presence or absence of preferential pH_i regulation is contingent on inducing a sufficiently severe pH_e reduction to influence pH_i; therefore, the treatments to impose acidoses and the sampling times were chosen to ensure a severe pH_e reduction. For a respiratory acidosis, hyperoxia was used as it has not been previously examined in *A. transmontanus*, unlike hypercarbia (see above references). The origin of the respiratory acidosis differs between hypercarbia and hyperoxia, whereby the former induces an acidosis due to increased external CO_2. The latter induces an acidosis arising from reduced ventilatory rate due to high environmental oxygen; thus, reducing CO_2 excretion and leading to an increase in metabolically produced CO_2 (Wood and LeMoigne, 1991), and unlike hypercarbia, hyperoxia does not reduce water pH, which may impair pH_e regulation in *A. transmontanus* (Shartau et al., 2017b). Metabolic acidoses were induced via exhaustive exercise, anoxia or air exposure, where the acidosis is generated intracellularly (via anaerobiosis), with the associated acid exported to the extracellular space. Treatments imposing metabolic acidoses often produce a mixed acid-base metabolic and respiratory acidosis (Kieffer et al., 1994; Wang et al., 1994). The response of *A. transmontanus* to these metabolic acidoses may also inform, to some degree, on the acid-base relevant effects of challenges such as catch and release fishing (McLean et al., 2016) and swimming/migration (Cocherell et al., 2011; Erickson et al., 2002; Geist et al., 2005).

2.2 Methods

2.2.1 Animal acquisition and holding

All experiments were performed at the International Centre for Sturgeon Studies (ICSS) at Vancouver Island University (VIU) using *A. transmontanus* (656 ± 181 g). All white sturgeon were maintained in large indoor flow-through tanks in dechlorinated City of Nanaimo tap water [61 μmol l^{-1} Na^+, 69 μmol l^{-1} Cl^- (City of Nanaimo, 2015), pH ~6.6-6.8 (Mojazi Amiri et al., 2009)] at ~15 ^oC under a simulated natural photoperiod and were fed daily to satiation. Food was withheld 48 h prior to experiments. All experiments

were approved both by the University of British Columbia and Vancouver Island University Animal Care Committees (animal care no: A11-0235; Animal Usage Protocol: 2014-02-R).

2.2.2 Experimental protocol

For all treatments, eight white sturgeon were randomly selected from the holding tank and placed in individual black plexi-glass boxes (24 L) with aeration in a re-circulating system (flow rate ~3 L min^{-1} per box, 15 ^{o}C; total water volume of system ~320 L) overnight prior to experiments. Confinement in darkened boxes has been suggested to not stress *A. oxyrinchus* Atlantic or *A. brevirostrum* shortnose sturgeon (Baker et al., 2005a); similarly, juvenile *Scaphirhynchus albus* pallid and hybrid *S. albus* × *S. platorynchus* shovelnose sturgeon have low physiological responses to severe confinement (Barton et al., 2000). Control fish were sampled following overnight holding; experimental manipulations are described below for each individual treatment. Sampling was staggered to ensure adequate time to euthanize, sample each animal and take blood measurements.

2.2.3 Respiratory acidosis

Hyperoxia induces a respiratory acidosis via the retention of metabolically produced CO_2. In this treatment, fish were held overnight, then aeration was stopped to the recirculating system, while aeration to individual tanks was maintained. All boxes were isolated from the recirculating system and rapid O_2 bubbling was initiated in the main header tank to increase O_2 tension to ~80 kPa PO_2 (~15 min). Once achieved, the boxes were re-connected to the recirculating system in a staggered fashion (~20 min), and P_wO_2 increased to the target tension of ~80 kPa PCO_2 within 15 min. Fish were then exposed to ~80 kPa P_wO_2 for 180 min to achieve a hyperoxic-induced respiratory acidosis of similar magnitude and duration as previous hypercarbic-induced respiratory acidoses (Baker et al., 2009a; Baker et al., 2015; Brauner et al., 2004).

2.2.4 Metabolic acidosis

Metabolic acidoses were induced via exhaustive exercise, anoxia or air exposure. White sturgeon subjected to exhaustive exercise were removed from tanks and subjected to a repeated exhaustive exercise protocol similar to the one used on armoured catfish (Harter et al., 2014). Fish were chased with a plastic stick for 5 min or until the fish was completely exhausted, allowed to rest for 15 min, then chased again until complete exhaustion, which occurred within 15 min.

In the anoxia treatment, after overnight holding, aeration was stopped to the recirculating system, while aeration to the individual tanks was maintained. All boxes were then isolated from the recirculating system and rapid N_2 bubbling was initiated in the main header tank to reduce O_2 tension to <1 kPa P_wO_2 (~15 min). Once achieved, the boxes were re-connected to the recirculating system in a staggered fashion (~20 min), and P_wO_2 decreased to the target tension of <1 kPa P_wO_2 within 15 min. Fish were continuously exposed to <1 kPa P_wO_2 for 5 min; following the anoxia exposure, the box was disconnected and aerated, returning P_wO_2 to >90% saturation within 2 min and fish were then allowed to recover until sampling (see below).

Acipenser transmontanus were exposed to air following overnight holding in individual tanks. In a staggered fashion, water was drained from the respective tank, then a damp cloth was placed over the fish to minimize desiccation and stress; air temperature was ~15 ^{o}C. After 45 min, the tank was filled with aerated water and the fish were allowed to recover until sampling.

Fish subjected to exhaustive exercise or anoxia were sampled at 15 or 120 min after the challenge, different time points were used to allow for any redistribution of acidoses and to assess those changes on pH. Due to limited fish numbers, fish subjected to air exposure were only sampled at 15 min post-exposure.

2.2.5 Blood sampling, tissue sampling and ions

At the time of sampling, the box in which the fish was held was isolated from the re-circulating system and anesthetic was added to the water (MS-222 0.3 g L^{-1} buffered with $NaHCO_3$) under vigorous aeration to avoid hypoxemia due to reduced ventilation. Once ventilation ceased (<3 min), each fish was turned ventral side up, while gills

remained submerged in aerated water and blood (3 mL) was drawn caudally via a lithium-heparin (1 g L^{-1})- rinsed syringe (5 mL syringe, 23 G1¼ needle) and placed on ice. Following this procedure, fish were killed via cephalic concussion and the following tissues were removed within 2-3 min, placed in aluminum foil and immediately placed in liquid N_2 in the following order: heart (gently squeezed and patted dry to remove any blood), liver, dorsal white muscle (left side, just posterior of the dorsal fin; skin and red muscle removed), and brain; tissues were stored longer term at -80 ^{o}C. Blood was divided into two aliquots. Blood pH and hematocrit (Hct) were measured from one aliquot; the other aliquot was centrifuged (3 min at 10,000 rpm) and plasma was removed for measurement of total CO_2 (TCO_2), [Cl^-] and [lactate].

Blood pH was measured using a Radiometer PHM 84 (Copenhagen, Denmark) connected to a Radiometer Analytical SAS pH electrode (GK2401C, Cedex, France) thermostated at 15 ^{o}C. RBC pH_i was measured using the freeze-thaw method as described by Zeidler and Kim (1977). Tissue pH_i was measured using the metabolic inhibitor tissue homogenate method (MITH; see Appendix for detailed description of this method) (Baker et al., 2009b; Portner et al., 1990). Plasma TCO_2 was measured using a total CO_2 analyzer (Corning model 965 Analyzer); the remaining plasma was used to measure [Cl^-] ions (HBI model 4425000; digital chloridometer). For determination of plasma [lactate], 200 µL 8% perchloric acid was added to 200 µL plasma and immediately frozen in LN_2 and stored at -80 ^{o}C until assayed for lactate via the method described by Bergmeyer (1983).

2.2.6 Calculations and statistical analysis

Plasma [HCO_3^-] and PCO_2 were calculated using TCO_2 and pH values described by Brauner et al. (2004). CO_2 solubility coefficient and the logarithmic acid dissociation constant (pK') for plasma were determined from Boutilier et al. (1984).

All values are expressed as mean ± s.e.m. throughout; N=8 for all treatments. Data were compared by Welch's t-test or where multiple treatments were evaluated, data were analyzed by an analysis of variance (ANOVA), followed by Tukey's or Dunnett's post hoc tests or if the data did not meet normality (Shapiro-Wilk normality test) or equal variance (Bartlett's test) assumptions a Kruskal-Wallis test followed by Dunn's multiple

comparison test was used ($P<0.05$). GraphPad Prism (v.5) was used for all statistical analyses and for preparation of figures.

2.3 Results

2.3.1 Extracellular acid-base status

The objective of all treatments was to induce a reduction in pH_e to examine pH changes in the tissues. Here, all treatments were successful in reducing pH_e, although the severity of pH_e reduction varied amongst acidoses (Fig. 2.1 and 2.2). A respiratory acidosis induced by 180 min hyperoxia exposure increased blood PCO_2 to 1 kPa PCO_2. This increase in blood PCO_2 led to a reduction in pH_e and an increase in plasma HCO_3^- immediately following this exposure (Fig. 2.1).

Acipenser transmontanus exercised to exhaustion experienced a large pH_e reduction (0.30 units) at 15 min post-exercise along with an increase in PCO_2, but no change in plasma HCO_3^- was observed. Compared to 15 min post-exercise, at 120 min, pH_e was unchanged, but PCO_2 and plasma HCO_3^- were lower (Fig. 2.2a). Exposure to anoxia reduced pH_e by only 0.1 units by 15 min post-anoxia and increased PCO_2 and plasma HCO_3^-; pH_e, PCO_2 and plasma HCO_3^- did not change by 120 min post-anoxia (Fig. 2.2b). Following 45 min air exposure, both blood PCO_2 and plasma $[HCO_3^-]$ increased, while pH_e decreased by 0.35 units (Fig. 2.2c).

2.3.2 Intracellular acid-base status

A hyperoxia-induced respiratory acidosis reduced RBC and muscle pH_i immediately following 180 min exposure; heart, liver and brain pH_i did not change (Fig. 2.3).

Exhaustive exercise reduced RBC, liver and muscle pH_i at 15 and 120 min post-exercise, while heart pH_i was only reduced at 120 min post-exercise; brain pH_i did not significantly change (Fig. 2.4a). Anoxia exposure reduced pH_i of RBC, liver and brain at 15 and 120 min post-exposure; muscle pH_i was reduced at 120 min post-exposure (Fig.

2.4b). Air exposure resulted in a reduction in RBC, liver and muscle pH_i at 15 min post-exposure (Fig. 2.4c).

2.3.3 Hematocrit, plasma [Cl⁻] and [lactate]

Exhaustive exercise induced the greatest change in these parameters relative to control values, where plasma [Cl^-], [lactate] and hematocrit all increased following exercise. Hematocrit also increased in the hyperoxia exposure, but not in anoxia or air exposure. Plasma [lactate] was elevated compared to controls after 15 min post-acidosis in anoxia, hyperoxia and air exposure. Plasma [Cl^-] did not change following anoxia, hyperoxia or air exposure (Table 2.1).

2.4 Discussion

The goal of this study was to investigate whether preferential pH_i regulation in white sturgeon is a general strategy of acid-base regulation, irrespective of the origin of the acid-base disturbance. Our results indicate that *A. transmontanus* preferentially regulate pH_i against acidoses of respiratory origin [hyperoxia (this study) and hypercarbia (Baker et al., 2009a)]; however, during the metabolic acidosis treatments (exhaustive exercise, anoxia and air exposure), which created mixed metabolic/respiratory acidoses, preferential pH_i regulation did not occur uniformly amongst the tissues. These results only partially support the hypothesis and indicate preferential pH_i regulation of tissues may occur selectively amongst various acidoses; this differs from *P. pardalis*, where preferential pH_i regulation was observed to be a general strategy of acid-base regulation (Brauner et al., 2004; Harter et al., 2014). Finally, this study demonstrates that responses to various acidoses may invoke different physiological responses; consequently, caution should be taken when extrapolating results amongst different types of acidoses.

2.4.1 White sturgeon preferentially regulate pH_i during respiratory acidoses

Preferential pH_i regulation appears to be a general strategy of acid-base regulation during respiratory acidoses in white sturgeon. During hyperoxia, blood PCO_2 increased

to approximately 1 kPa and reduced pH_e by 0.15 units at 180 min; this was slightly less severe than previous studies using hypercarbia in *A. transmontanus* where exposure to 1.5 kPa PCO_2 reduced pH_e by 0.2 units at 6 h (Fig. 2.5a) (Baker et al., 2009a). The pH_e reduction in Figure 5a is similar to other studies exposing different sized *A. transmontanus* to 1.5 kPa PCO_2 for 3 h (Baker and Brauner, 2012; Baker et al., 2011; Shartau et al., 2017b), suggesting there is minimal pH difference between these time points and that fish size is unlikely to affect the magnitude of pH change. Similar to pH_e, RBC pH_i was reduced by 0.11 units, indicating that the hyperoxia-induced acidosis was sufficiently severe to reduce pH in a highly buffered tissue lacking capacity for pH regulation and is consistent with the response during hypercarbia (Brauner et al., 2004; Harter et al., 2014). Unlike the blood or RBC, pH_i of heart, brain and liver did not change, indicating they were protected during hyperoxia (Fig. 2.3). The degree of pH_i regulation appears to be less than during hypercarbia as heart, brain and liver exhibit an increase in pH_i following 6 h exposure to 1.5 kPa PCO_2 (Fig. 2.5b) (Baker et al., 2009a). Interestingly, muscle pH_i was reduced during hyperoxia (Fig. 2.3) but not during hypercarbia (Fig. 2.5b). The reduction in muscle pH_i may be associated with tissue anaerobiosis as suggested by the increase in plasma lactate during hyperoxia (Table 2.1). The reason for the increase in plasma lactate during hyperoxia is unknown but muscle lactate increased during 24 h exposure to 2 kPa PCO_2 (Baker and Brauner, 2012).

Few studies have measured pH_e and pH_i concurrently during hyperoxia. Hyperoxia induces an increase in blood PCO_2 arising from the retention of metabolically produced CO_2 of ~1 kPa PCO_2. When *Oncorhynchus mykiss* (rainbow trout) were exposed to 72 h hyperoxia, Hobe et al. (1984) found that pH_e, and white muscle and whole body pH_i were reduced; similarly Wood and LeMoigne (1991) observed that pH_e and pH_i of brain and muscle were reduced. Not surprisingly, hypercarbia and hyperoxia induce a similar increase in internal blood PCO_2, and also induce similar changes in pH_e (Gilmour and Perry, 1994) and pH_i (Shartau, unpublished observation) in rainbow trout. Respiratory acidoses induced by hyperoxia and hypercarbia appear to result in similar pH changes in both *O. mykiss* and *A. transmontanus*, which exhibit coupled pH regulation and preferential pH_i regulation, respectively; as the acidoses have similar origins, they may affect pH similarly in these fishes.

2.4.2 Tissue pH_i is differentially protected following metabolic acidoses

Acipenser transmontanus do not uniformly protect pH_i following the development of a metabolic acidosis induced by exhaustive exercise, anoxia or air exposure. As expected RBC pH_i was reduced in all treatments, however, there were also reductions in liver and muscle pH_i. In contrast, heart pH_i remained unchanged in all treatments at 15 min post-exposure and brain pH_i was protected following exhaustive exercise and air exposure (Fig. 2.4). The response of *A. transmontanus* to the metabolic acidoses indicates that their pattern of response differs from that of *P. pardalis*, which completely protect brain, heart and liver against exhaustive exercise and anoxia (Harter et al., 2014). Compared to respiratory acidoses, *A. transmontanus* exhibit greater variability with respect to pH regulation amongst metabolic acidoses; this may be due to differences in oxygen availability and demand during conditions leading to metabolic acidoses. Acid production during metabolic acidoses originates from the tissue due to increased anaerobic metabolism following reduced oxygen supply and/or increased oxygen demand. Consequently, anaerobic metabolism will be recruited to different degrees as oxygen supply and demand may change differentially amongst tissues; the observed pH_i reduction is greatest in white muscle, while heart pH_i remains well protected.

Acipenser transmontanus tightly regulate heart pH_i during metabolic acidoses (exhaustive exercise, anoxia and air exposure), to a degree similar to that accomplished during respiratory acidoses (hyperoxia and hypercarbia). Heart pH_i may be more tightly regulated given a) the importance of the heart in O_2 transport and b) that changes in pH_i may lead to electrical disturbances disrupting cardiac function (Vaughan-Jones et al., 2009). Preferential regulation of heart pH_i may thus be important to maintain metabolic activity and avoid issues related to cardiac function, particularly during acute acidoses. During hypercarbia (Baker et al., 2011; Hanson et al., 2009) and combined hypercarbia/hypoxia (Shartau et al., 2016b), cardiac performance is maintained along with protection of heart pH_i. During exercise, heart pH_i is maintained during exercise of *Hemitripterus americanus* (sea raven) (Milligan and Farrell, 1986) and *Parophrys vetulus* (lemon sole) (Wright et al., 1988). Interestingly, *A. transmontanus* exhibited a small reduction in heart pH_i 120 min post-exercise; this reduction may have been due to the

redistribution of the acidosis from other tissues and/or the persistent elevation of plasma lactate which can influence pH_i (Vaughan-Jones et al., 2009).

Brain pH_i was protected following exhaustive exercise and air exposure but not anoxia, where it was reduced by ~0.07 units (Fig. 2.4). Tight regulation of brain pH_i likely ensures proper metabolic function when sufficient energy supplies exist; however, the brain is one of the most metabolically active tissues and highly sensitive to perturbation of energy supply (Soengas and Aldegunde, 2002). In low O_2 conditions, anaerobic metabolism is insufficient to maintain brain ATP level; thus, in hypoxic/anoxic tolerant animals, the fall in ATP production is partially compensated for by anaerobic ATP production and supplemented by suppression of ATP use (metabolic depression) which together assist brain survival during hypoxia/anoxia (Hochachka, 1986; Nilsson, 2001; Soengas and Aldegunde, 2002). Consequently, reduction in brain pH_i during anoxia may represent a survival mechanism, where in fact, maintaining pH_i at normoxic levels would be maladaptive due to the energy required, possibly leading to reduced hypoxia/anoxia tolerance and survival. The difference in O_2 availability for aerobic metabolism may explain in part why the response in anoxia differs from exercise and air exposure.

As exercise requires increased white muscle activity, it would be expected to be the site of the greatest rate of anaerobiosis due to the higher energy demand required during exercise. This in turn could result in high rates of ATP hydrolysis, outpacing ATP production, and consequently creating an excess of protons (Robergs et al., 2004). Consequently, muscle tissue overall would be expected to exhibit the greatest reduction in pH_i during exhaustive exercise. In this study, muscle pH_i was reduced by 0.44 units following exhaustive exercise, the largest reduction in pH_i of any tissue measured; it was also associated with a large increase in plasma lactate (Table 2.1). Reduced muscle pH_i following anoxia and air exposure may have been a result of a more general reduction in O_2 availability, prompting an increase in the rate of anaerobic metabolism despite reduced activity.

The reduction in liver pH_i across all metabolic acidoses could indicate that the liver is less capable of pH_i regulation than other tissues, but this response may also be associated with handling of metabolic waste products (e.g. lactate) (Richards, 2011). For

example, in *Carassius auratus* (goldfish), both the liver and white muscle act as a store of glycogen to support whole animal metabolism during anaerobic metabolism (Jibb and Richards, 2008). Indeed, following exercise and anoxia, *C. auratus* reduce muscle pH_i, possibly due to the accumulation metabolic wastes such as H^+ and lactate arising from the depletion of glycogen stores (Mandic et al., 2008). During hypoxia, goldfish liver and muscle tissues have reduced pH_i and increased lactate, with pH_i reduction attributed to regulation of protein synthesis and metabolic reduction (Jibb and Richards, 2008). Reduction in liver and muscle pH are also observed in *Squalus acanthias* (dogfish) during recovery from severe hypoxia (Zimmer and Wood, 2014); these changes parallel those observed in *A. transmontanus* here (Fig. 2.4, Table 2.1).

2.4.3 Preferential pH_i regulation may be a general strategy of acid-base regulation in A. transmontanus – but not all tissues are protected all the time

Taken together, these finding illustrate that *A. transmontanus* have different responses to acid-base regulation depending on the source and tissue which the acidosis originates. During respiratory acidoses, pH_i is preferentially regulated in critical tissues, but following metabolic acidoses induced by exhaustive exercise, anoxia and air exposure, only some tissues are protected (e.g. heart). This pattern differs from *P. pardalis*, which preferentially regulate pH_i following respiratory (hypercarbia) (Brauner et al., 2004) and metabolic acidoses (exhaustive exercise, anoxia) (Harter et al., 2014); although hyperoxia and air exposure were not been examined in that study. The reason for differences in pH_i regulation between these two species is unclear. However, it may be due to differences in species specific capacity for pH_i regulation or it could indicate that acid-base strategies differ between environments (i.e. tropical versus temperate rivers) and that in general, tropical fishes, may have a greater capacity for pH_i regulation to deal with differences of their environment; uncovering these differences is worthy of further investigation.

2.4.4 Conclusions

Sturgeon demonstrate remarkable resilience to a variety of stressors including severe hypercarbia (Baker et al., 2009a), hyperoxia (Bagherzadeh Lakani et al., 2013;

Shartau et al., 2017b), hypoxia (Baker et al., 2005a; Crocker and Cech, 1997; Maxime et al., 1995), exercise (Baker et al., 2005b; Cocherell et al., 2011; Kieffer et al., 2001), aerial exposure (Brauner and Berenbrink, 2007), salinity (Allen et al., 2014; McEnroe and Cech, 1985; Mojazi Amiri et al., 2009; Shaughnessy et al., 2015) and fisheries related stressors (McLean et al., 2016). This study may provide insight into sturgeon acid-base regulation during these stressors, as even though the treatments imposed in this study were severe, they may occasionally be encountered naturally. For example, migrating *A. transmontanus* may be subjected to acute periods where they exercise to near exhaustion (Cocherell et al., 2011; Geist et al., 2005), and catch and release fishing causes them to exercise to exhaustion while enduring some degree of air exposure (angling events may last between 30 seconds to over 2 hours) (McLean et al., 2016). Additionally, sturgeon are often reared for aquaculture and in these settings, hyperoxia may be encountered and exceed >40 kPa PO_2 (Bagherzadeh Lakani et al., 2013; Espmark and Baeverfjord, 2009), while hypoxia as low as 4 – 10 kPa PO_2 may be experienced by Atlantic (Gunderson, 1998) and white (Crocker and Cech, 1997) sturgeon in estuaries. While tolerance and survival of these stressors requires a multifaceted physiological response, these results indicate that in *A. transmontanus* during the acidoses examined, the robust capacity for pH_i regulation may contribute to their overall tolerance during these acid-base challenges. The molecular and cellular mechanism(s) that underlie preferential pH_i regulation are unknown and currently under investigation (Shartau et al., 2017b). Further research into this area should provide insight into the differential response of pH_i regulation during acidoses in sturgeon, and possibly inter-specific differences in acid-base regulatory strategy.

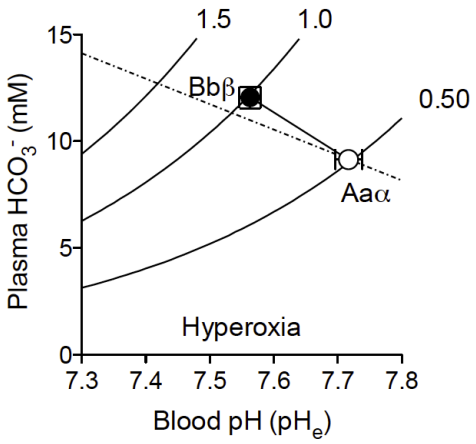


Figure 2.1: Effect of a hyperoxia-induced respiratory acidosis in *Acipenser transmontanus* white sturgeon on blood acid-base status. Blood pH (pH_e) and plasma [HCO_3^-] are presented on a pH-HCO_3^- plot. *A. transmontanus* were exposed to 80 kPa PO_2 for 180 min and sampled either prior to (control; ○) or at the end of the exposure (●). Values are presented as means ± s.e.m.; N=8. Significant differences (P<0.05) are indicated by different uppercase letters (pH_e), lowercase letters (blood PCO_2) and Greek letters (plasma HCO_3^-). Dashed line indicates the blood non-bicarbonate buffer line (-11.9 mM HCO_3^- pH $unit^{-1}$) for *A. transmontanus* as determined by Baker et al. (2009a).

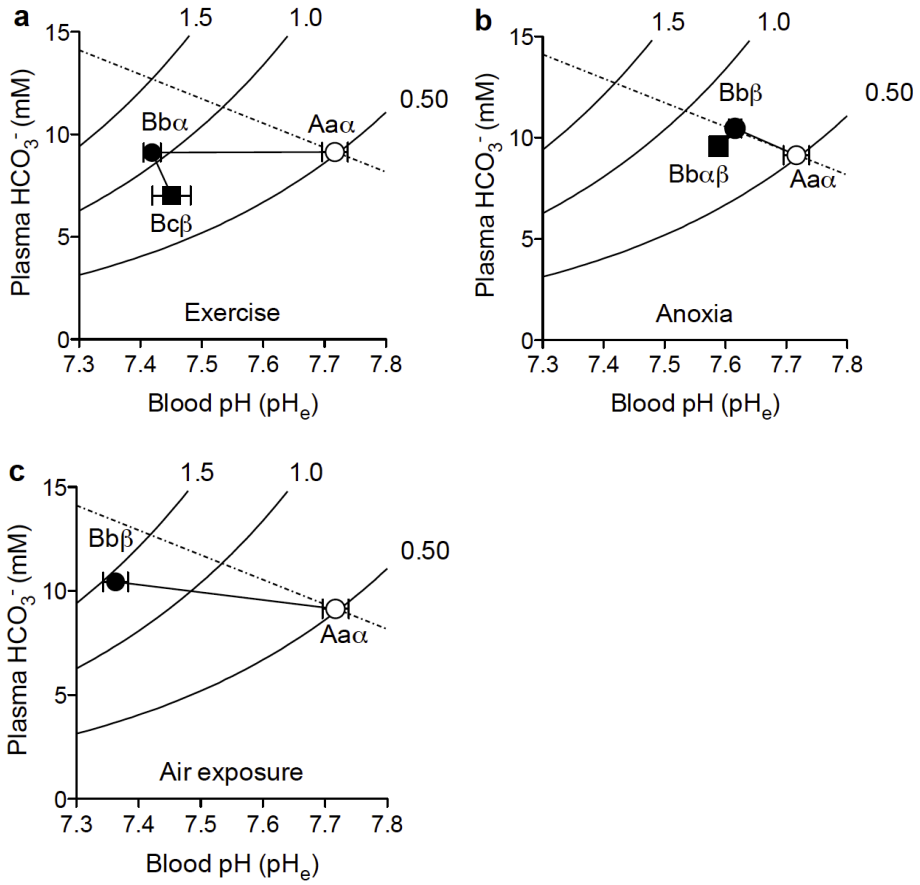


Figure 2.2: Effect of metabolic acidoses in *Acipenser transmontanus* (white sturgeon) on blood acid-base status. Blood pH (pH_e) and plasma [HCO_3^-] are presented on a pH-HCO_3^- plot. White sturgeon were subjected to either exhaustive exercise (a), anoxia (5 min exposure; b) or air exposure (45 min; c). Fish were sampled either prior to exposure (control; ○), or following exposure after a 15 (●) or 120 minutes (■; except air exposure which was only sampled at 15 min) recovery period. Values are presented as means ± s.e.m.; N=8. Significant differences (P<0.05) are indicated by different uppercase letters (pH_e), lowercase letters (blood PCO_2) and Greek letters (plasma HCO_3^-). Dashed line indicates the blood non-bicarbonate buffer line (-11.9 mM HCO_3^- pH $unit^{-1}$) for white sturgeon as determined by Baker et al. (2009a).

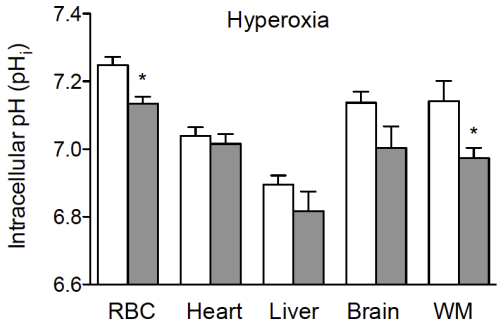


Figure 2.3: Effect of a hyperoxia-induced respiratory acidosis in *Acipenser transmontanus* (white sturgeon) on intracellular pH (pH_i) of red blood cells (RBC), heart, liver, brain and white muscle (WM). *Acipenser transmontanus* were exposed to 80 kPa PO_2 for 180 min and sampled either prior to (control; open bar) or at the end of the exposure (shaded bar). Values are presented at means ± s.e.m. Asterisk indicate significant differences from control (P<0.05).

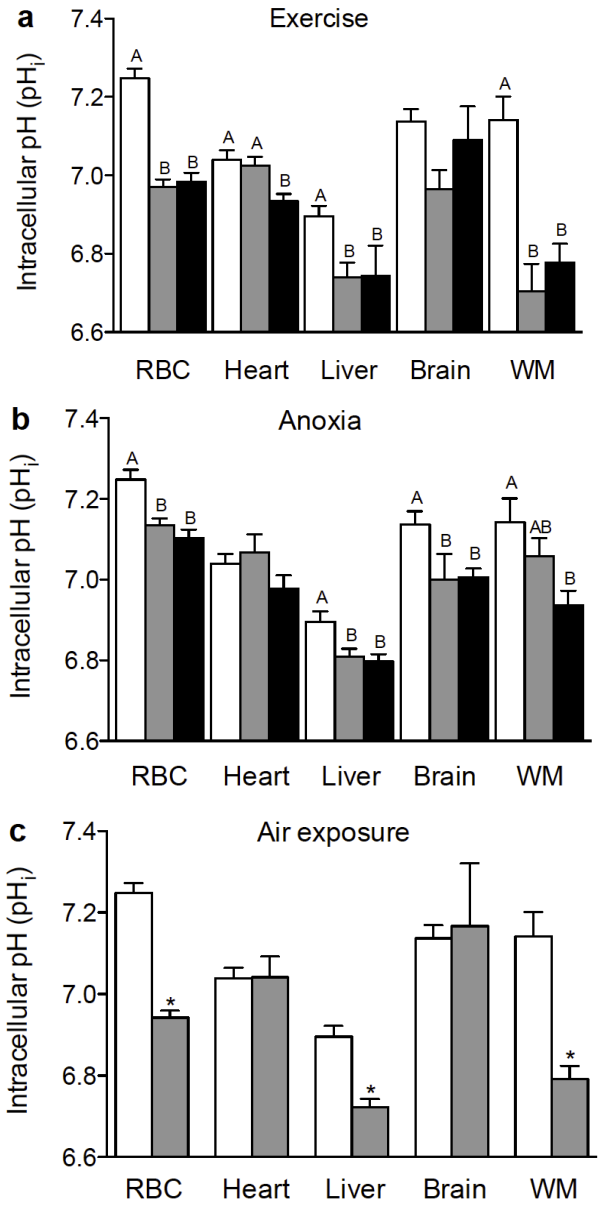


Figure 2.4: Effect of metabolic acidoses in *Acipenser transmontanus* (white sturgeon) on intracellular pH (pH_i) of red blood cells (RBC), heart, liver, brain and white muscle (WM). *Acipenser transmontanus* were subjected to either exhaustive exercise (a), anoxia (5 min exposure; b) or air exposure (45 min; c). Fish were sampled either prior to exposure (control; open bar), or following exposure after a 15 (shaded bar) or 120 minutes (closed bar; except air exposure) recovery period. Values are presented at means ± s.e.m. Significant differences are indicated by different letters, except for air exposure where an asterisk denotes difference from control (P<0.05).

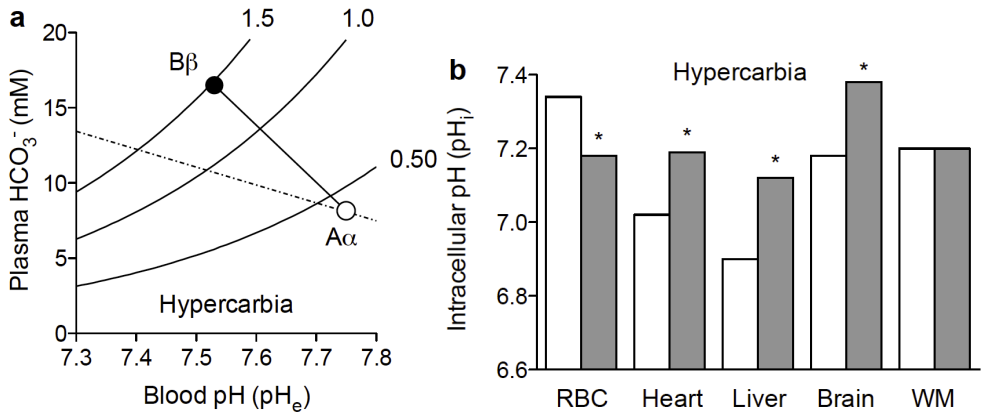


Figure 2.5: Effect of a hypercarbic-induced respiratory acidosis in *Acipenser transmontanus* (white sturgeon) on blood and tissue acid-base status following a 6 h exposure to 1.5 kPa PCO_2. Blood pH (pH_e) and plasma [HCO_3^-] are presented on a pH-HCO_3^- plot; fish were sampled either prior to (control; ○) or at the end of the exposure (●) (a). Dashed line indicates the blood non-bicarbonate buffer line (-11.9 mM HCO_3^- pH $unit^{-1}$) for *A. transmontanus* as determined by Baker et al. (2009a). Intracellular pH (pH_i) of red blood cells (RBC), heart, liver, brain and white muscle (WM) when sampled either prior to (control; open bar) or at the end of the exposure (shaded bar) (b). Data is re-plotted from Baker et al. (2009a). Significant differences (P<0.05) for pH_e, plasma HCO_3^- and pH_i, as determined by Baker et al. (2009a), are indicated by uppercase letters, Greek letters and asterisks, respectively.

Table 2.1: Effect of various treatments inducing an acidosis on hematocrit, plasma [Cl^-] and [lactate] in *Acipenser transmontanus* (white sturgeon).

Treatment	Post-exposure time (min)	Hematocrit (%)	Plasma [Cl^-] (mM)	Plasma [lactate] (mM)
Control		**27±0.5**	**125±4**	**2.9±0.4**
Hyperoxia	0	29±1*	129±1	4.6±0.5*
Exercise	15	35±1*	140±5*	6.2±0.5*
	120	33±1*	130±1	6.2±0.5*
Anoxia	15	29±1	133±5	4.1±0.2*
	120	29±1	125±2	3.8±0.3
Air exposure	15	26±2	129±3	5.0±0.3*

Values are indicated as means ± s.e.m., N=8; significant differences from control are indicated by an asterisk (P<0.05).

Chapter 3: Preferential Intracellular pH Regulation May Represent a Common Strategy of Acid-Base Regulation Amongst CO_2 Tolerant Fishes

3.1 Introduction

Large transient increases in CO_2 (hypercarbia) are common in many aquatic environments and pose challenges for acid-base regulation in fishes (Brauner and Baker, 2009; Hasler et al., 2016; McNeil and Sasse, 2016; Shartau and Brauner, 2014). When subjected to acute hypercarbia, fishes will experience an increase in blood PCO_2 as CO_2 diffuses and equilibrates across the gills, which leads to a reduction in blood and extracellular pH (pH_e), referred to as a respiratory acidosis. The most common response observed in fishes [e.g. *Eptatretus stoutii* (Pacific hagfish) (Baker et al., 2015), *Scyliorhinus stellaris* (dogfish) (Heisler et al., 1988), *Gadus morhua* (Atlantic cod) (Larsen et al., 1997), *Oncorhynchus mykiss* (rainbow trout) (Hobe et al., 1984), *Conger conger* (conger eel) (Toews et al., 1983)] is that pH_e is compensated by a net increase in plasma [HCO_3^-] in exchange for Cl^-, with the gills playing the primary role in compensation (Brauner and Baker, 2009). Depending on the severity of the acidoses and the ionic composition of the water (Larsen and Jensen, 1997), complete pH_e compensation typically occurs within 24-72 h. Putative limits on the elevation in plasma [HCO_3^-] appear to prevent complete pH_e compensation during exposure to acute PCO_2 >2 kPa (Baker et al., 2015; Brauner and Baker, 2009; Heisler, 1984). Changes in pH_e are often associated with qualitatively similar changes in intracellular pH (pH_i) as the two are typically coupled, referred to as 'coupled pH regulation'; thus, recovery of pH_e is important for pH_i recovery (Shartau et al., 2016a). Failure to maintain acid-base homeostasis will negatively impact fitness in environments subject to severe acute hypercarbia as deviations from normal physiological pH values can affect molecular charge, altering the structure and function of biological macromolecules, and, ultimately, reducing whole-animal performance (e.g. reduce heart and skeletal muscle contractility,

alter metabolic pathways, and disrupt cellular signalling and processes such as volume regulation) (Boron, 2004; Occhipinti and Boron, 2015; Putnam and Roos, 1997).

Many of the world's freshwater fishes inhabit hypercarbia-prone environments. Globally, the average PCO_2 of stream and river systems is ~0.3 kPa (*ca*. 3100 μatm), about 8-fold above current atmospheric levels (Raymond et al., 2013); some tropical freshwater lakes and rivers may experience a range of CO_2 tensions up to ~2 kPa (20,249 μatm) (Cole et al., 1994) and ~5.5 kPa (54,270 μatm) (de Fátima F L Rasera et al., 2013), respectively. In many aquatic systems, there may be localized point sources for high PCO_2 which may further increase PCO_2 due to vegetative covering and respiration of aquatic life (Hasler et al., 2016); in the Amazon River basin PCO_2 may reach 8 kPa (Furch and Junk, 1997; Heisler, 1984) and the presence of CO_2 vents could produce PCO_2 >50 kPa (Sorey et al., 2000). Within manmade aquatic systems, PCO_2 can easily rise beyond 2-4 kPa PCO_2 within recirculating aquaculture systems and aquaculture ponds (Crocker and Cech, 1996; Damsgaard et al., 2015). Additionally, modifications to river systems via creation of dams have the potential to create environments prone to experiencing hypercarbia (de Faria et al., 2015). There is also interest in controlling movement of invasive fish species using CO_2 as a barrier by increasing regional PCO_2 levels to as high as ~5-11 kPa (100-200 mg/L) (Dennis et al., 2016; Kates et al., 2012; Tierney, 2016). In the marine environment, there are natural CO_2 seeps at some locations which create localized hypercarbia (Basso et al., 2015; Melzner et al., 2009), thus affecting marine life (Brauner and Baker, 2009; Hawkins, 2004; Lackner, 2003). There is a great variation in water PCO_2 worldwide that species have adapted to, or have to deal with due to anthropogenic influences, and often PCO_2 exceeds the putative limits for pH_e regulation. However, relatively little is known about how fish tolerate and compensate for these high CO_2 levels.

Some groups of fishes may be especially well adapted to severe hypercarbia as evident in tropical freshwater environments which have a high diversity of species. Tropical environments contain 56% of watersheds with high fish species diversity (Val et al., 2005), and this is exemplified by the species rich Rio Negro, an acidic ion-poor Amazon River tributary, that contains over 1000 species (Gonzalez et al., 2017). A number of basal euteleostom fishes also reside in hypercarbic-prone habitats, with the

majority being bimodal breathers, which may increase the likelihood of experiencing a respiratory acidosis (Shartau and Brauner, 2014). This suggests many fishes are well adapted to responding to hypercarbia; indeed, Regan et al. (2016) observed that brain function in *Pangasianodon hypophthalmus* (striped catfish), a tropical bimodal breather, is adapted for the hypercarbic waters of the Mekong River and is disrupted during exposure to normocapnia. Coupled pH_e/pH_i regulation may be constrained in high CO_2 environments, yet little is known about how fishes tolerate and survive severe acute hypercarbia.

In the few fishes studied to date that tolerate hypercarbia well above 2 kPa PCO_2, it appears tolerance is associated with the ability to completely regulate pH_i of heart, brain, liver and muscle, despite a large, often uncompensated, reduction in pH_e (preferential pH_i regulation) (Shartau et al., 2016a). Preferential pH_i regulation has been demonstrated in *Synbranchus marmoratus* (marbled swamp eel) (Heisler, 1982), *Pterygoplichthys pardalis* (armoured catfish) (Brauner et al., 2004) and *Acipenser transmontanus* (white sturgeon) (Baker et al., 2009a) exposed to PCO_2 ranging from 3-6 kPa. This strategy of acid-base regulation, at least in *A. transmontanus*, appears to provide near instantaneous pH_i regulation [(Baker, 2010); reviewed in (Shartau et al., 2016a)] and does not exert a whole animal metabolic cost (Baker and Brauner, 2012). In *P. pardalis*, preferential pH_i regulation is a general strategy of pH regulation, used during both respiratory and metabolic acid-base challenges (Harter et al., 2014); however, in *A. transmontanus*, pH protection may be tissue specific during metabolic acidoses (Shartau et al., 2017a). It has been previously hypothesized that preferential pH_i regulation confers exceptional CO_2 tolerance in fishes, as it may be the strategy by which they are able to cope with a severe acute respiratory acidosis. Protection of tissue pH during acidoses is important as large pH_i reduction in *O. mykiss* muscle following exhaustive exercise is believed to be responsible for increased mortality following exercise (Wood et al., 1983). Lower heart pH_i is associated with reduced heart contractibility, and thus, cardiac performance may be reduced leading to diminished O_2 delivery (Vaughan-Jones et al., 2009). A reduction in brain pH_i due to hypercarbia has an anesthetic affect, causing a loss of equilibrium which may lead to mortality (Yoshikawa et al., 1994). By preferentially regulating pH_i during a hypercarbic-induced respiratory acidosis, the above fishes may

avoid these damaging affects and thus, inhabit and/or travel through environments experiencing short-term severe hypercarbia. As few fish species have been shown to preferentially regulate pH_i (Shartau et al., 2016a), it remains unclear if this strategy is widely used, or is confined to a select few basal actinopterygian and air breathing fishes (Shartau and Brauner, 2014); that few species are known to use this pattern of acid-base regulation is likely a result of few studies exposing fishes to severe hypercarbia while simultaneously measuring pH_e and pH_i.

We hypothesize that CO_2 tolerant fishes utilize preferential pH_i regulation and that fishes from a number of different families and orders use this strategy of acid-base regulation. The main objective of this study was to conduct a survey of preferential pH_i regulation and CO_2 tolerance in a group of phylogenically diverse fishes that included 20 fishes originating from three continents (North America, South America and Africa), representing 11 orders, and range from basal vertebrates (e.g. lamprey) to derived actinopterygians (e.g. tilapia). We first devised a CO_2 tolerance assay to assess acute CO_2 tolerance (Series I). Next, acid-base response of various fish species were examined using terminal pH_e/pH_i sampling following severe acute hypercarbia ranging from 1.5-6 kPa PCO_2, depending on their CO_2 tolerance (Series II). Finally, Series III used the CO_2 tolerance assay to indirectly determine preferential pH_i regulation in a number of other fish species to gain a broader understanding of the phylogenetic distribution of preferential pH_i regulation. Together, these objectives provide the most comprehensive examination of acid-base regulation during acute hypercarbia in fishes conducted to date.

3.2 Methodology

3.2.1 Animal acquisition and holding

In this study, 20 species of fish were used and experiments were conducted as follows. From September 2012 to November 2013 measurements were made on the following species at the University of British Columbia, Vancouver, BC, Canada): *Oncorhynchus mykiss* (rainbow trout) (250-400g) from Miracle Springs Inc. (Mission, BC, Canada); *Entosphenus tridentatus* (Pacific lamprey) (~200g) caught Fall 2013 on the

Nechako river near Prince George, BC, Canada; *Oncorhynchus kisutch* (coho salmon) (~100g) UBC aquaculture facility (Vancouver, BC, Canada); *Oreochromis niloticus* X *mossambicus* X *hornorum* (tilapia hybrid) (~300-400g) from Redfish Ranch (Courtenay, BC, Canada). Experiments with *Acipenser transmontanus* (white sturgeon) (~200g) were conducted at the International Centre for Sturgeon Studies, Vancouver Island University, Nanaimo, BC, Canada using fish reared in their facility.

Experiments were conducted at the Instituto Nacional de Pesquisas da Amazônia/INPA (Manaus, AM, Brazil) with fish caught in the Rio Negro near Manaus and transferred to a holding facility at INPA in 2008: *Hoplosternum littorale* (tamoata), *Brycon amazonicus* (matrinxa), *Colossoma macropomum* (tambaqui) and *Astronotus ocellatus* (oscar). In 2013 the following species were caught from the wild and investigated at INPA: *Potamotrygon sp*. (freshwater ray) (~50-80g), *Lepidosiren paradoxa* (South American lungfish) ~ 300-1500g, *Synbranchus marmoratus* (marbled swamp eel) ~ 50-150g, *Electrophorus electricus* (electric eel). The following fish were obtained from local fish farms and transferred to facilities at INPA, *Arapaima gigas* ~ 75-100g, *C. macropomum*, *Astronotus ocellatus* (oscar), and *Pterygoplichthys pardalis* (armoured catfish) ~100g.

Experiments with the following farm reared species were conducted at the South Farm Aquaculture research facility at Mississippi State University (Starkville, MS, USA) in March 2013: *Polyodon spathula* (American paddlefish) ~ 150-400g, *Atractosteus spatula* (alligator gar) ~ 400-1000g, *Ictalurus punctatus* (channel catfish) ~ 100 – 200g, *Ictalurus punctatus* X *I. furcatus* (channel X blue catfish) ~ 100 – 200g. Experiments with the following species were conducted at the University of North Texas (Denton, TX, USA) with fish caught from nearby lakes/rivers in November 2012: *Lepisosteus oculatus* (spotted gar) ~ 300-700g, and *I. punctatus* ~ 50-200g.

Typically, animals were kept for at least 2 weeks in appropriate tanks under standard conditions of food, temperature and natural photoperiod before experiments; however, some fish were wild caught and only held for 72 h due to constraints on animal housing. Fish were not fed at least 48 h before experiments.

3.2.2 Series I: CO_2 tolerance assay

To determine the optimal rate of CO_2 increase for assessing CO_2 tolerance, fish species that are known to use different acid-base regulatory strategies in response to hypercarbia were used, *O. mykiss* (coupled pH regulation) and *A. transmontanus* (preferential pH_i regulation) (Shartau et al., 2016a). Fish were randomly selected from the holding tank and placed in individual black plexi-glass boxes (24 L) with aeration in a re-circulating system (flow rate ~3 L min^{-1} per box, 15 ^{o}C; total water volume of system ~320 L) overnight prior to experiments. Fish were then exposed to progressively increasing levels of hypercarbia at a rate of 1, 2 or 4 kPa PCO_2 h^{-1}. Water PCO_2 was monitored to ensure PCO_2 increased at the desired rate for the duration of exposure using a thermostated (15 ^{o}C) Radiometer PCO_2 electrode (E5036) (output, Radiometer PHM 73). Fish were continuously observed for loss of equilibrium (LOE), which was used as the end point to indicate their CO_2 tolerance. LOE was defined as the inability to maintain dorsoventral orientation. Once LOE was reached, fish were immediately removed from the box and placed in a normocarbic, normoxic recovery tank; fish were monitored for at least 48 h after hypercarbia exposure and there were no mortalities.

3.2.3 Series II: Strategy of acid-base balance during severe acute hypercarbia

The strategy of acid-base regulation used during severe acute hypercarbia in fishes was determined by first subjecting them to the CO_2 tolerance assay at a rate of 2 kPa PCO_2 h^{-1}, as was previously determined to be a suitable rate in Series I. The assay was targeted to ensure fish would tolerate one of three desired PCO_2 test exposures (1.5, 3 or 6 kPa). The highest CO_2 tension fish could tolerate was used in order to observe pH_i during maximal pH_e depression; as pH_i only changes by approximately 1/3 of pH_e in coupled pH regulators, (due to a lower starting pH_i value and greater tissue buffer value) larger reductions in pH_e allow for a more accurate determination of whether fishes preferentially regulate pH_i. Once the we determined the CO_2 tension that fish species of interest could tolerate, we performed the following experiment to assess whether they utilize either coupled pH regulation or preferential pH_i regulation.

Fish were acclimated individually for 24 h in black plexi-glass boxes in the system described above; this period is sufficient to allow recovery from handling stress in

sturgeon (Baker et al., 2005b; Barton et al., 2000). Normocarbic fish were terminally sampled immediately (see below) following this acclimation period (control group). Other fish were then exposed to 3 h hypercarbia at either 1.5, 3 or 6 kPa PCO_2, depending on the fish species. We also examined the response of an extremely hypercarbia tolerant species (based on series I), *C. macropomum*, to more severe hypercarbia of 20 kPa PCO_2 as we were interested if CO_2 tolerance at this high CO_2 tensions is associated with preferential pH_i regulation as is the case at lower, but still severe hypercarbia levels.

The 3 h time point was chosen to sample fish in Series II because at this time pH_i is typically maximally reduced and pH_i compensation is more rapid than pH_e compensation. Furthermore, this provides sufficient time for CO_2 to increase in all tissues resulting in pH_i depression if fish are coupled pH regulators or no change in pH_i if they are preferential pH_i regulators (Shartau et al., 2016a). Hypercarbia was achieved by bubbling a mixing tank with preset rates of air and 100% CO_2 using Sierra Instruments mass flow controllers. Water PCO_2 was measured with a PCO_2 electrode to confirm target CO_2 tensions; water O_2 levels remained >80% saturation.

At the time of sampling, each box was isolated from the recirculation system and anesthetic was added to the water (MS-222 0.3 g/L buffered with $NaHCO_3$) while hypercarbic gas bubbling was maintained to minimize changes in blood PCO_2 and avoid hypoxemia due to reduced ventilation. Once ventilation ceased (<3 min), each fish was turned ventral side up, while gills remained submerged in aerated water and blood (~2-3 mL) was drawn from the caudal vein into a lithium-heparin (1 g L^{-1}) rinsed syringe (3 mL syringe, 23 G1¼ needle) and placed on ice. Following this procedure, fish were killed via cephalic concussion and cervical dislocation and tissues (0.5-1.0 g) were removed within 2-3 min, wrapped in aluminum foil and immediately flash frozen in liquid N_2. Tissues were sampled in the following order: heart (gently squeezed and patted dry to remove any excess blood), liver, dorsal white muscle (left side, just posterior of the dorsal fin; skin and red muscle removed), and brain; tissues were stored longer term at -80 ^{o}C. Blood was divided into two aliquots. Blood pH and hematocrit (Hct) were measured from one aliquot; the other aliquot was centrifuged (3 min at 10,000 rpm) and plasma was removed for measurement of total CO_2 (TCO_2) and [Cl^-].

Blood pH was measured using a Radiometer PHM 84 (Copenhagen, Denmark) connected to a thermostated Radiometer Analytical SAS pH electrode (GK2401C, Cedex, France). RBC pH_i was measured using the freeze-thaw method as described by Zeidler and Kim (Zeidler and Kim, 1977). Tissue pH_i was measured using the metabolic inhibitor tissue homogenate method (MITH; see Appendix for detailed description of this method) (Portner et al., 1990) and validated for use in fish by Baker et al. (2009b). Plasma TCO_2 was measured using a total CO_2 analyzer (Corning model 965 Analyzer); the remaining plasma was used to measure $[Cl^-]$ ions (HBI model 4425000; digital chloridometer). Plasma $[HCO_3^-]$ and PCO_2 were calculated using TCO_2 and pH values described by Brauner et al. (2004). CO_2 solubility coefficient and the logarithmic acid dissociation constant (pK') for plasma were determined from Boutilier et al. (1984).

3.2.4 Series III: CO_2 tolerance to infer pattern of pH regulation

To conduct a more rapid and non-lethal assessment of pH regulation in fishes, we used the CO_2 tolerance assay to determine CO_2 tolerance in various species, as determined by point of LOE. Fish were placed individually in boxes, allowed to acclimate 24 h and then exposed to a target rate of increase of 2 kPa PCO_2 h^{-1} until LOE was reached or in the case of *E. tridentatus*, *Potamotrygon sp*., *P. pardalis* and *S. marmoratus* when these fishes became unresponsive to gentle prodding with a plastic stick. As in Series I, once LOE was reached, fish were removed and allowed to recover. Fishes reaching LOE >8 kPa PCO_2 are considered to be preferential pH_i regulators as *O. mykiss* did not tolerate PCO_2 beyond this tension at any other rates of increase (Fig. 3.1), and thus suggests fish tolerant to CO_2 tensions greater than this are preferential pH_i regulators.

3.2.5 Calculations and statistical analysis

All values are expressed as means ± s.e.m. throughout. Data were compared by Welch's t-test or where multiple treatments were evaluated, data were analyzed by analysis of variance (ANOVA), followed by Tukey's post hoc test. If the data did not meet the assumptions of normality (Shapiro-Wilk normality test) or equal variance (Bartlett's test), a Kruskal-Walis test followed by Dunn's multiple comparison test was

used ($P<0.05$). GraphPad Prism (v.5) was used for statistical analyses and preparation of figures.

3.3 Results

3.3.1 Series I: Development of a CO_2 tolerance assay

To determine a rate of CO_2 increase to assess acute CO_2 tolerance, *O. mykiss* and *A. transmontanus* were exposed to progressively increasing levels of hypercarbia at a rate of 1, 2 or 4 kPa PCO_2 h^{-1}. The mean PCO_2 at which LOE occurred in rainbow trout was 5.5 ± 0.3, 4.8 ± 0.3 and 2.7 ± 0.2 kPa PCO_2 at rates of 1, 2 and 4 kPa PCO_2 h^{-1}, respectively, while that in *A. transmontanus* was 22.1 ± 2.2, 14.6 ± 2.3 and 6.3 ± 1.8 kPa PCO_2, respectively (Fig. 3.1). Within species, the PCO_2 LOE at 4 kPa PCO_2 h^{-1} was lower relative to the other rates ($P<0.01$); there was no difference between 1 and 2 kPa PCO_2 h^{-1}. Comparison between species at the different rates of PCO_2 increase indicated that *A. transmontanus* had a higher PCO_2 LOE at 1 and 2, but not 4 kPa PCO_2 h^{-1} ($P<0.01$). No mortalities occurred in the 72 h following LOE in fish allowed to recover in normocarbia.

3.3.2 Series II: Survey of pH_i regulation

Acute hypercarbia exposure to 1.5 kPa PCO_2 in *P. spathula*, 4 kPa PCO_2 in *H. littorale*, *B. amazonicus*, *C. macropomum* and *A. ocellatus*, and 6 kPa PCO_2 in *L. oculatus*, *A. spatula*, *I. punctatus*, and *Oreochromis sp.* reduced both pH_e and RBC pH_i as expected; the sole exception was *A. ocellatus* RBC where there was limited sample size (n=2). In contrast, pH_i increased in the heart of *L. oculatus*, *H. littorale*, *B. amazonicus*, *C. macropomum* and *A. ocellatus* ($P<0.05$), *A. spatula* liver, *I. punctatus* brain and *Oreochromis sp.* white muscle (Fig. 3.2); no other statistically significant changes were observed in other tissues. Similarly, exposure of *C. macropomum* to 20 kPa PCO_2 severely reduced pH_e from 7.729 ± 0.03 to 6.896 ± 0.024 pH units. Red blood cell (RBC) and white muscle pH_i was reduced but there are no statistically significant changes in

heart, liver or brain pH_i (Fig. 3.2G). Results in all of these fish species are consistent with the capacity for preferential pH_i regulation (Shartau et al., 2016a).

Exposure of *O. mykiss* to 3 kPa PCO_2 also resulted in the expected reduction in pH_e and RBC pH_i. In line with those changes, pH_i was reduced in heart, liver, brain, and white muscle (Fig. 3.2F); these results are in agreement with previous studies and consistent with use of coupled pH regulation (Shartau et al., 2016a).

Where measured, there were no significant changes in plasma Cl^-, or osmolarity in any fishes. Only *O. mykiss* experienced an increase in hematocrit during exposure to 1.5 and 3 kPa PCO_2 while *P. spathula* exhibited a reduction (Table 3.1).

3.3.3 Series III: CO_2 tolerance

Using the Series I CO_2 tolerance assay at a rate of 2 kPa PCO_2 h^{-1}, the mean PCO_2 at which LOE occurred were determined for the following species: *E. tridentatus* - 14.6 ± 0.4 kPa, *Potamotrygon sp.* 11.1 ± 0.2 kPa, *P. spathula* 2.7 ± 0.5 kPa, *A. transmontanus* 14.5 ± 2.3 kPa, *A. gigas* 24.4 ± 1.0 kPa, *I. punctatus* 8.4 ± 0.1 kPa, *I. punctatus* X *I. furcatus* 7.4 ± 0.2 kPa, *P. pardalis* 14.0 ± 0.9 kPa, *O. mykiss* 4.8 ± 0.3 kPa, *O. kisutch* 5.5 ± 0.7 kPa, *A. ocellatus* 13.9 ± 0.6 kPa, and *Oreochromis sp.*12.6 ± 0.5 kPa (Table 3.2). Several fish species were highly CO_2 tolerant and their tolerance exceeded our ability to measure CO_2 which was limited to 26.7 kPa PCO_2 (200 torr PCO_2); these fishes did not reach LOE at a rate of 2 kPa PCO_2 h^{-1}: *S. marmoratus*, *L. paradoxa*, *C. macropomum*, *E. electricus* (n=2). Immediately following LOE, fish were transferred to normocarbic waters and no mortalities were observed in the subsequent 48 h of recovery.

3.4 Discussion

Our objective was to investigate the prevalence of preferential pH_i regulation in phylogenically diverse fishes to understand how they maintain acid-base homeostasis during severe acute hypercarbia. We show that preferential pH_i regulation is used by fishes tolerant of severe acute hypercarbia, and that it is present in species from numerous phylogenetic orders; thus, likely representing a general strategy of acid-base regulation

amongst fishes (Fig. 3.3). These results support our hypothesis that CO_2 tolerant fishes use preferential pH_i regulation. However, we also show that preferential pH_i regulation is not sufficient to confer CO_2 tolerance (Fig. 3.3; Table 3.2) as was hypothesized by Brauner and Baker (2009). This study demonstrates that preferential pH_i regulation may be an important and widespread trait allowing fishes to tolerate, and thus survive hypercarbia in diverse aquatic environments.

3.4.1 Use of CO_2 assay for tolerance to acute hypercarbia

Previous studies examining CO_2 tolerance have generally exposed fishes to a certain PCO_2 and recorded the time at which behaviour changes or LOE is achieved (Hasler et al., 2017; Hayashi et al., 2004; Kates et al., 2012); consequently, these methodologies do not provide an estimate as to the maximal acute PCO_2 fish can tolerate, nor do they suggest an appropriate rate of CO_2 increase to investigate hypercarbia tolerance. This study shows that 1 and 2 kPa PCO_2 h^{-1} may provide the best estimate of acute CO_2 tolerance as there was no difference in the PCO_2 at which LOE were reached, whereas at 4 kPa PCO_2 h^{-1}, the LOE PCO_2 was significantly lower (Fig. 3.1). It is uncertain why the faster rate of PCO_2 increase resulted in a lower LOE PCO_2 but could be a consequence of the rapid CO_2 induced acidification outpacing the cellular defenses to mitigate the acidosis. Additionally, the lack of difference between *O. mykiss* and *A. transmontanus* LOE PCO_2 at 4 kPa PCO_2 h^{-1} prevents differentiating between strategies of acid-base regulation. Where multiple runs of this assay were conducted using different individuals (*L. oculatus*, *P. spathula*, *I. punctatus*; limited fish numbers precluded us from repeating the CO_2 tolerance assay in all species), we observed that the PCO_2 at which LOE occurs is consistent (Table 1). Recently, Hasler et al. (2017) demonstrated that hypercarbia tolerance is a repeatable, and likely a heritable, trait within individuals of *Micropterus salmoides* (largemouth bass).

Using this CO_2 tolerance assay as an indicator of acute tolerance may not accurately reflect natural environmental exposures, nor is it likely to indicate the maximal capacity of fishes to compensate for gradual, chronically induced hypercarbia. However, the rapid acute CO_2 exposure in this assay does provide an approximation of fishes' ability to protect critical tissues against rapid acidification, which is thought to be the

cause of LOE (Yoshikawa et al., 1994), and we propose indicates the presence of preferential pH_i regulation which in the sturgeon heart has been shown to be virtually instantaneous during CO_2 exposure [Baker, 2010, reviewed in (Shartau et al., 2016a)]. In fishes where pH_i was measured along with CO_2 tolerance, it was observed that in those that were more tolerant of hypercarbia than *O. mykiss* (which use coupled pH regulation), are also preferential pH_i regulators. In addition to *A. transmontanus, A. spatula*, *L. oculatus*, *I. punctatus*, *P. pardalis*, *A. ocellatus*, *Oreochromis sp*., *S. marmoratus*, *C. macropomum* are CO_2 tolerant as indicated by the CO_2 tolerance assay developed here and direct pH measurements have confirmed their ability for preferential pH_i regulation during severe acute hypercarbia. Therefore, CO_2 tolerance, as demonstrated in this assay, may provide an assessment regarding the capacity for fishes to protect pH_i, and thus, use preferential pH_i regulation. The association between high CO_2 tolerance and preferential pH_i regulation was corroborated with pH measurements in a number of species; however, the relationship between low CO_2 tolerance and coupled pH regulation only occurred in *O. mykiss*, and thus should be investigated more thoroughly among other coupled pH regulators.

3.4.2 Acid-base regulation during hypercarbia

Studies measuring acid-base status in fishes exposed to acute hypercarbia have typically observed concurrent pH_e and pH_i reductions (Brauner and Baker, 2009; Shartau et al., 2016a). However, most of these studies have investigated fish exposed <2 kPa PCO_2, and those subjecting fishes to more severe hypercarbia have not typically investigated how they maintain acid-base homeostasis (Shartau et al., 2016a). Fishes dependent on coupled pH_e/pH_i regulation appear to be limited to compensating pH_e at PCO_2 <2 kPa due to putative limits on plasma HCO_3^- [the so-called "HCO_3^- concentration threshold" (Heisler, 1984)], which may be associated with preventing hypochloremia, as pH_e compensation is associated with a net increase in plasma HCO_3^- in equimolar exchange for plasma Cl^- (Baker et al., 2015; Brauner and Baker, 2009; Heisler, 1984). As many fishes inhabit environments where CO_2 may greatly exceed 2 kPa PCO_2 and likely experience large, frequent oscillations in CO_2 (Furch and Junk, 1997; Gonzalez et al., 2017; Heisler, 1984; Val et al., 2005), the use of coupled pH regulation is likely

insufficient for survival in these habitats, particularly in species rich regions such as the Amazon and Mekong river basins. In contrast preferential pH_i regulation appears to offer an advantageous strategy of acid-base regulation during acute CO_2 exposure, allowing fishes to protect pH_i against at least PCO_2 >15 kPa (Fig. 3.2G) in at least one species, which is quite remarkable.

Survival during severe acute acidoses may depend on protecting pH_i, not pH_e as mortality in marine fishes following exposure to severe acute hypercarbia is believed to be due reduced heart pH_i (Hayashi et al., 2004). In the latter, pH_i was not measured, but it was suggested O_2 supply was impaired as cardiac output dropped due to reduced cardiac contractility stemming from reduced cardiac pH (Vandenberg et al., 1994). Similarly, reduced brain pH may be responsible for the anesthetic effect in common carp *Cyprinus carpio* causing them to lose equilibrium (Yoshikawa et al., 1994). During an exercise-induced metabolic acidosis, reduced muscle pH was hypothesized to be the cause of post-exercise mortality in *O. mykiss* (Wood et al., 1983). The capacity for pH_i regulation in those fishes may be insufficient to protect against acidoses, thus leading to deleterious changes in pH_i that ultimately affect whole animal performance (see introduction). Irrespective of their ability to compensate pH_e, the cellular dysfunction accompanied by pH_i reduction renders these fishes sensitive to severe acid-base challenges. In contrast, species maintaining pH_i during these acid-base challenges, particularly in critical tissues such as the heart, appear to be resilient to a range of respiratory and metabolic acidoses. For example, *P. pardalis* (Harter et al., 2014) and *A. transmontanus* (Shartau et al., 2017a) tolerate a range of acidoses which may be largely due to their capacity for preferential pH_i regulation. Tolerance of severe acute hypercarbia in this study is likely due to the exceptional capacity for pH_i regulation and is best exemplified in this study by pH_i protection of heart and brain in *C. macropomum* during exposure to 15 kPa PCO_2 (Fig. 3.2G).

3.4.3 Preferential pH_i regulation in fishes

Measurements of pH_e and pH_i in Series II reveals several species preferentially regulate pH_i during acute severe hypercarbia and the CO_2 assay suggests several other species also may have this ability. Use of the CO_2 tolerance assay without pH

measurements may not consistently infer the strategy of acid-base regulation if fish are sensitive to CO_2 as preferential pH_i regulation alone does not appear sufficient to confer CO_2 tolerance; this is demonstrated in *P. spathula* which fully protected pH_i despite having a relatively low CO_2 tolerance (2.7±0.5 kPa PCO_2) (Fig. 3.2A; Table 3.2). The basis for this low tolerance in *P. spathula* is uncertain, although it may be due, in part, to their high P_{50} and high MO_2 (Aboagye and Allen, 2014; Aboagye and Allen, 2017); thus, reductions in Hb-O_2 affinity due to Bohr/Root effects may hinder O_2 uptake and lead paddlefish to experience hypoxemia despite complete water O_2 saturation.

A greater degree of certainty is possible regarding the strategy of pH regulation when fishes are tolerant to high CO_2 as the [HCO_3^-] threshold putatively confers a physiological limit to pH_e regulation; consequently, fishes more tolerant than *O. mykiss*, where this limit has been demonstrated numerous times, are most likely to use preferential pH_i regulation. The association between high CO_2 tolerance and preferential pH_i regulation is reinforced by the now numerous examples of highly CO_2 tolerant fishes using preferential pH_i regulation. Use of preferential pH_i regulation is made all the more likely by the putative ionoregulatory disturbances that would occur if pH_e was compensated during severe hypercarbia. For example, complete pH_e compensation in *C. macropomum* with blood PCO_2 of 15 kPa would require a plasma [HCO_3^-] of *ca.* 250 mM; thus, complete pH_e compensation is not possible due to the ensuing changes in plasma osmolarity and ion balance that would occur. Even if compensation was desirable, typical freshwater teleost osmolarity is approximately 262-274 mM and plasma [Cl^-] is 125-132 mM (Table 3.1) (Edwards and Marshall, 2013); consequently, there is insufficient Cl^- to exchange for HCO_3^-, and even partial compensation would require a near total change in plasma ionic composition. Use of preferential pH_i regulation does not preclude pH_e compensation during hypercarbia exposure in all fishes as *H. littorale*, *B. Amazonicus*, *C. macropomum*, and *A. ocellatus*, all exhibited complete or partial pH_e compensation following 24 h hypercarbia exposure (data not shown). This is different than the response observed in *P. pardalis* which do not compensate pH_e following 96 h exposure to 4 kPa PCO_2 (Brauner et al., 2004), yet is similar to the *P. hypophthalmus* in the Mekong which compensate pH_e by 48 h at *ca.* 4 kPa PCO_2 (Damsgaard et al., 2015) while preferentially regulating pH_i (R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong,

M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong, and C.J.B., unpublished observations). Compensation of pH_e by these fishes generally conforms to the limits of the putative [HCO_3^-] threshold and this may indicate a preference to preserve whole animal acid-base homeostasis when possible.

Previously, preferential pH_i regulation had only been identified in three fishes (Baker et al., 2009a; Brauner et al., 2004; Heisler, 1982); we now show, via direct and indirect measurements, that another 15 species use preferential pH_i regulation during severe acute hypercarbia (Fig. 3.3). That preferential pH_i regulation is a rare pattern of acid-base regulation among vertebrates (Brauner et al., 2004) is unlikely, but rather it may be an ubiquitous strategy given the putative limits to pH_e regulation and the species richness in hypercarbic habitats. When previous studies (Baker et al., 2009a; Brauner et al., 2004; Heisler, 1982), unpublished observations [*Amia calva*, reviewed in (Brauner and Baker, 2009) and *P. hypophthalmus*, reviewed in (Shartau et al., 2016a)] and this study, are considered, there are 16 fish species likely to use preferential pH_i regulation, representing 9 euteleostomi fish orders, as well as an elasmobranch and agnathans (Fig. 3.3); suggesting preferential pH_i regulation is both a widely distributed and widely used strategy of acid-base regulation.

3.4.4 Preferential pH_i regulation: a strategy for expansion into hypercarbic environments?

The effect of hypercarbia in the context of anthropogenic climate change on fishes has been well studied; however, these current and future PCO_2 increases are greatly surpassed by existing natural CO_2 variations in many aquatic systems. The severe hypercarbic conditions in many environments pose challenges to acid-base regulation, however, this still remains an area ripe for investigation. Preferential pH_i regulation likely represents a key adaptation for survival in high CO_2 environments as an inability to protect pH_i is likely the proximate cause of mortality during, and following severe acidoses (Hayashi et al., 2004; Shartau et al., 2017a; Wood et al., 1983; Yoshikawa et al., 1994). Hypercarbia tolerance may have been an important selective pressure for niche expansion in aquatic habitats, particularly in the tropics, which may partially explain the tremendous species richness seen in these regions (e.g. Amazon and Mekong rivers);

additionally, hypercarbia, along with aquatic hypoxia, may have been a selective pressure for the evolution of air breathing (Ultsch, 1987; Ultsch, 1996). Aerial respiration in fishes typically increases blood $P\mathrm{CO_2}$ as (1) hypoxic waters are often simultaneously hypercarbic, and (2) $\mathrm{CO_2}$ release still largely occurs at the gills, and gill ventilation is typically reduced during air breathing (Shartau and Brauner, 2014); thus, $\mathrm{CO_2}$ tolerance conferred by preferential $\mathrm{pH_i}$ regulation may have been instrumental for the evolution of air breathing (Brauner and Baker, 2009; Shartau and Brauner, 2014).

In summary, this study is the most comprehensive investigation to date examining how fishes respond to severe acute respiratory acidoses. Here, 20 fishes originating from three continents (North America, South America and Africa), representing 11 orders, which include 17 families and 20 genera (Betancur-R et al., 2013), are investigated for their response to hypercarbia; these species range from basal vertebrates (e.g. lamprey) to derived actinopterygians (e.g. tilapia). This study demonstrates that preferential $\mathrm{pH_i}$ regulation is a widely used strategy to survive and tolerate $\mathrm{CO_2}$ tensions ranging from 3-20 kPa $P\mathrm{CO_2}$. As acid-base regulation is intimately associated with proper physiological functioning and ultimately survival, understanding how fishes (and vertebrates) co-opted preferential $\mathrm{pH_i}$ regulation to thrive in challenging environments may provide insight into key evolutionary transitions in vertebrates, such as the evolution of air breathing and the transition from water to land.

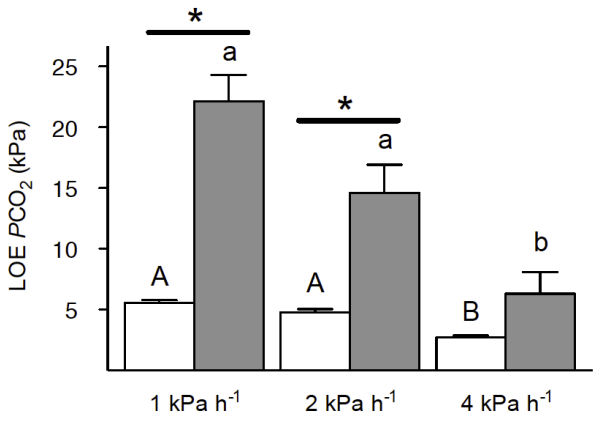
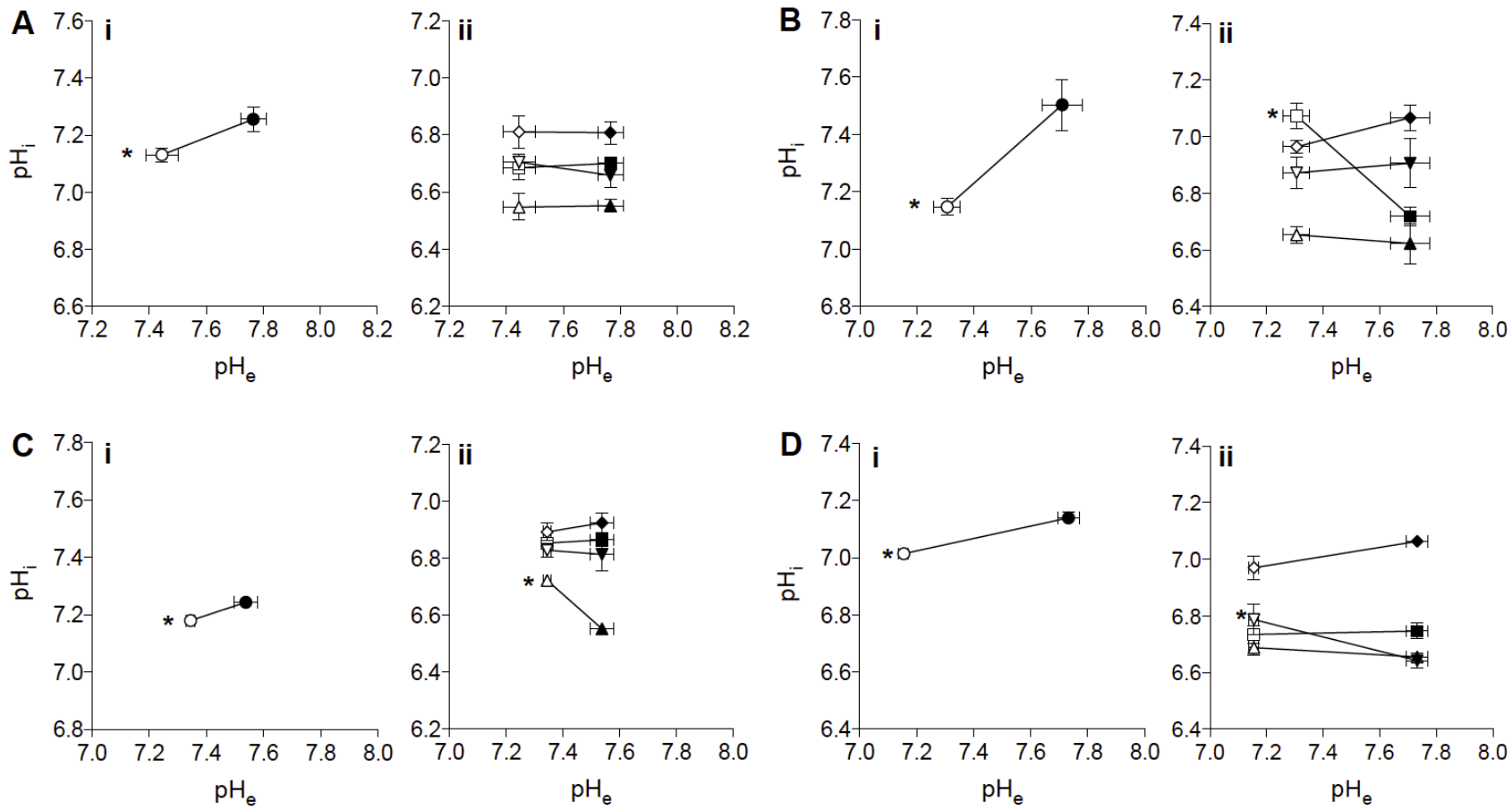


Figure 3.1 Bar plot of CO_2 tensions at loss of equilibrium in *Oncorhynchus mykiss* (open bars) and *Acipenser transmontanus* (grey bars) when subjected to a progressive increase in PCO_2 at 1, 2 or 4 kPa h^{-1}. Mean ± s.em. Significant differences due to rate of CO_2 increase within species are indicated by letters that differ (uppercase – *O. mykiss*; lowercase – *A. transmontanus*) ($P<0.05$). Differences between *O. mykiss* and *A. transmontanus*, which use coupled pH_e/pH_i and preferential pH_i regulation, respectively, at each rate of CO_2 increase are indicated by an asterisk ($P<0.05$).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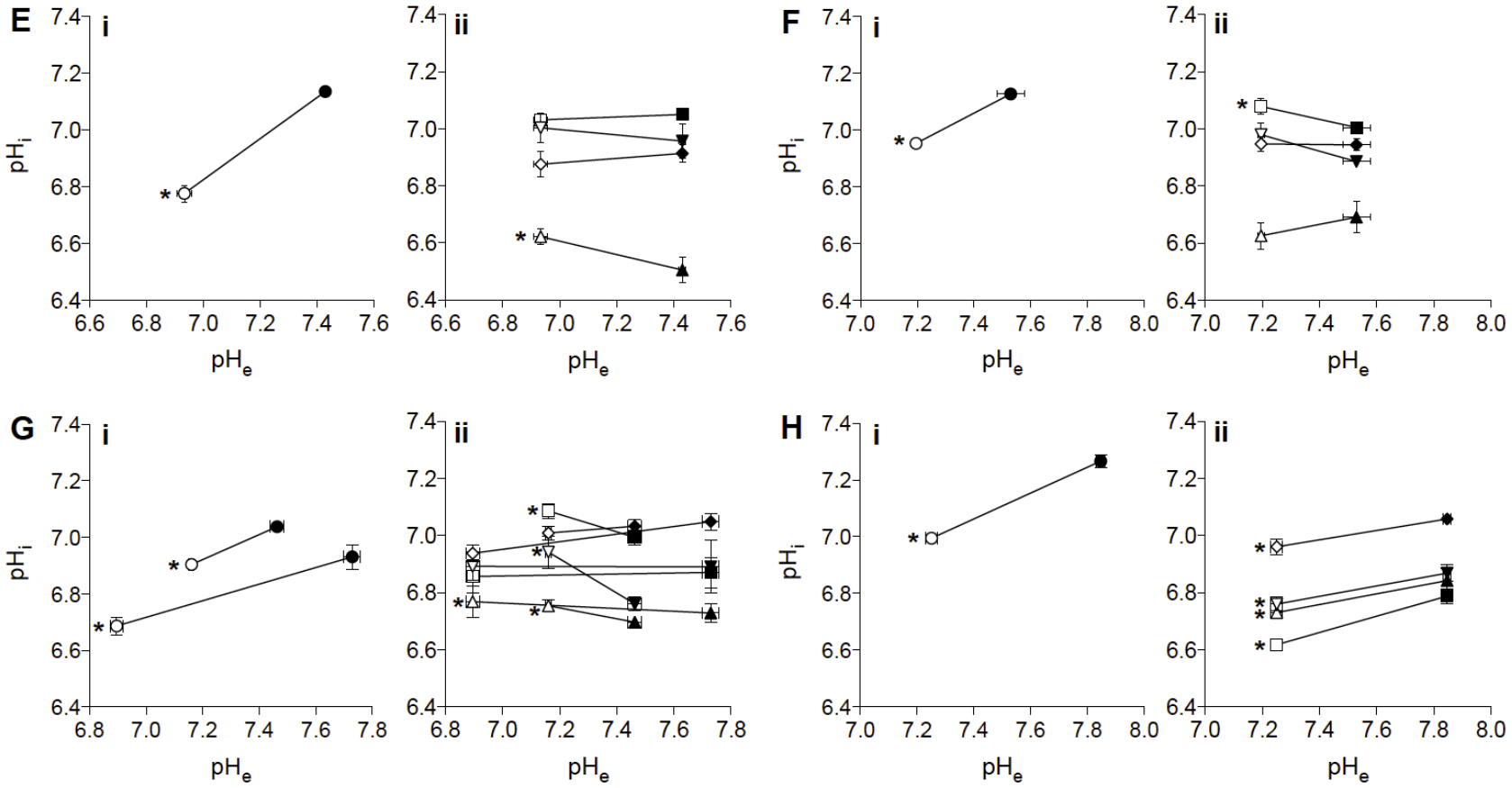
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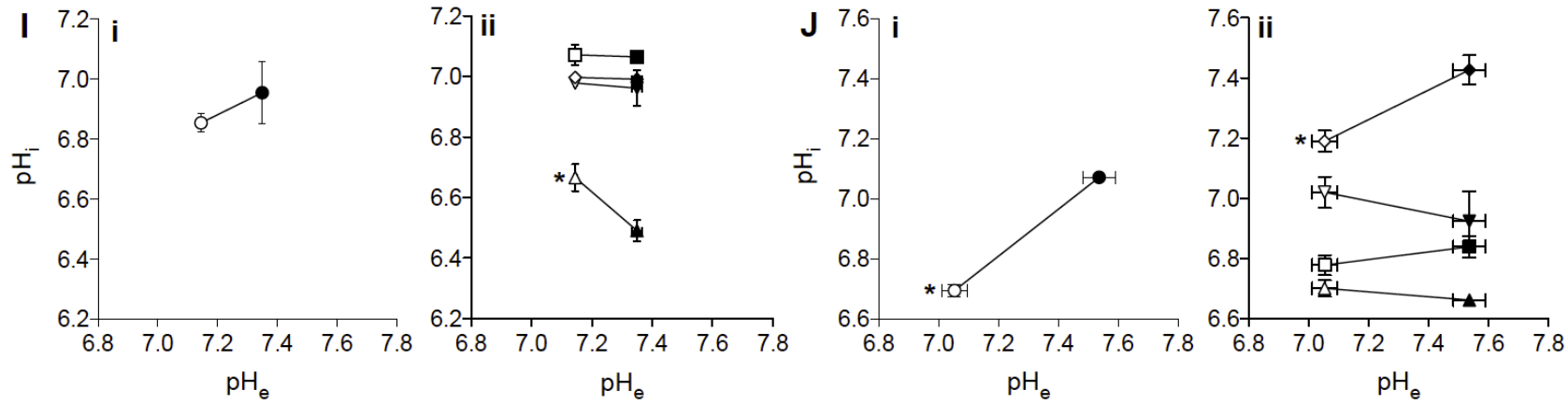

Figure 3.2: Effect of 3 h exposure to elevated CO_2 on blood and tissue acid-base status in 10 different fish species. The relationship between extracellular pH (pH_e) and intracellular tissue pH (pH_i) for each species is shown on two panels. Red blood cell (RBC) pH_i is plotted separately in the first panel for each species (i) as it is expected to be reduced during hypercarbia as RBCs generally appear to lack the capacity for pH_i regulation but possess high intracellular buffer capacity; thus, RBC pH_i demonstrate that the acidosis is sufficiently severe to reduce pH_i in a tissue unable to regulate pH_i and acts an internal control for the presence of an intracellular acidosis. In the second panel (ii) for each species, pH_i of heart (squares), liver (triangles), brain (inverted triangles) and white muscle (WM; diamonds) is plotted; sampling occurred at 0 (closed symbols) and 3 h (open symbols). Fish were subjected to hypercarbia for 3 h depending on their CO_2 tolerance as follows: *Polyodon spathula* (1.5 kPa PCO_2; A), *Lepisosteus oculatus* (6 kPa PCO_2; B), *Atractosteus spatula* (6 kPa PCO_2; C), *Ictalurus punctatus* (6 kPa PCO_2; D), *Hoplosternum littorale* (4 kPa PCO_2; E), *Brycon amazonicus* (4 kPa PCO_2, F), *Colossoma macropomum* (4 and 20 kPa PCO_2, G), *Oncorhynchus mykiss* (3 kPa PCO_2; H), *Astronotus ocellatus* (4 kPa PCO_2, I), *Oreochromis sp.* (6 kPa PCO_2; J). Significant differences between time points are indicated for each tissue by an asterisk (two-way t-test, $P<0.05$). In all species, pH_e was significantly reduced at 3 h ($P<0.05$) and pH_i of heart, brain, liver and white muscle were not reduced at any point except in *O. mykiss*; this is indicative of preferential pH_i regulation in all fishes except *O. mykiss* which exhibited coupled pH regulation.

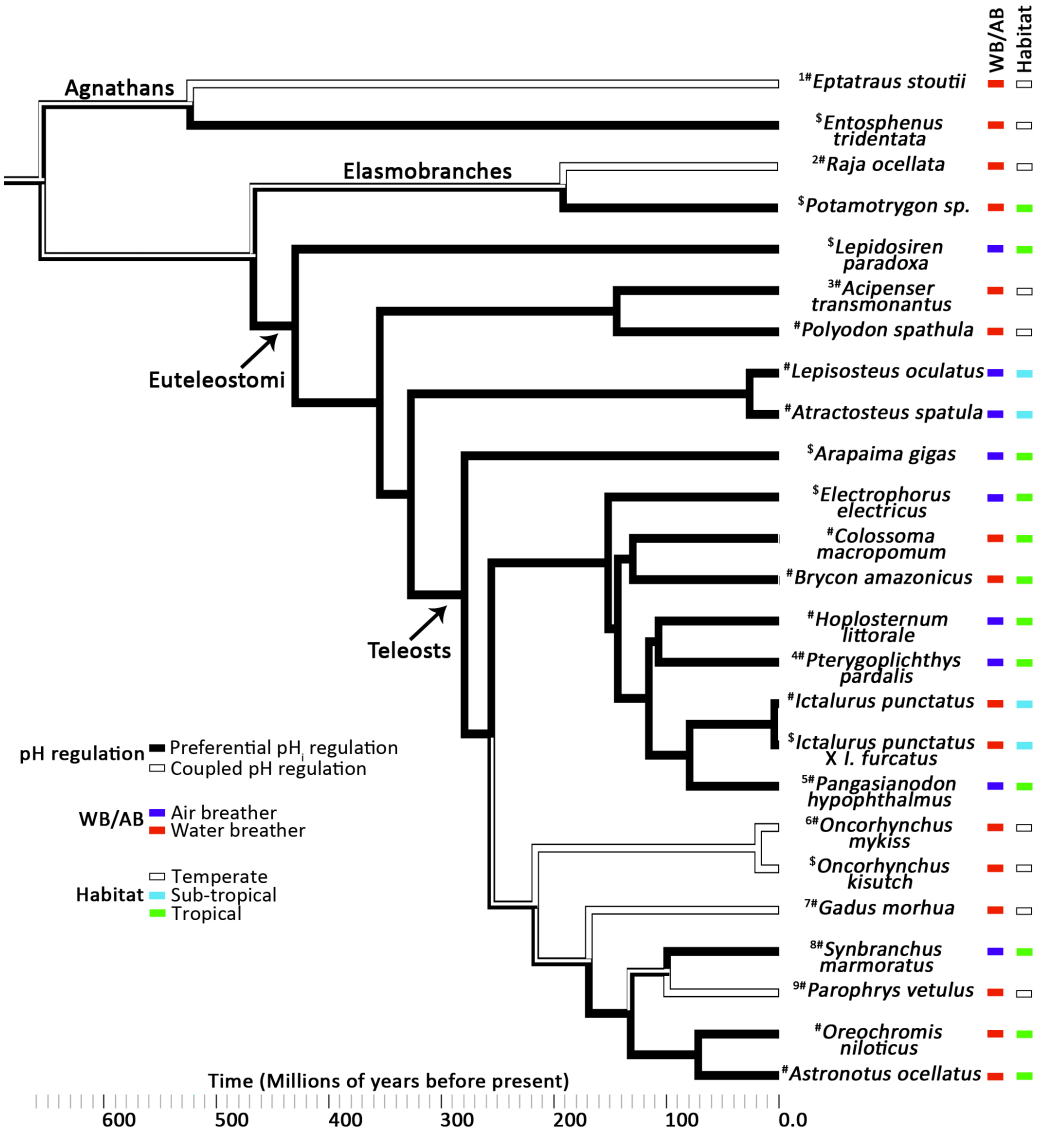

Figure 3.3: Evolution of preferential pH_i regulation and coupled pH_e/pH_i regulation amongst adult fishes exposed to an acute (<48 h) respiratory acidosis of >1 kPa blood $P\mathrm{CO_2}$. Pattern of acid-base regulation [preferential pH_i regulation (black branches) or coupled pH regulation (white branches)] was determined directly via pH measurements or indirectly via CO_2 tolerance, which are indicted by superscript # or $, respectively. Adjacent to species names it is indicated whether they are water or air breather, and the habitat of their primary geographical zone is listed (temperate, sub-tropical or tropical). Ancestral states for preferential pH_i regulation were reconstructed by likelihood using Mesquite (Maddison and maddison). Unless specified, all species were examined in this chapter; references are indicated below and correspond to superscript numbers: 1(Baker et al., 2015), 2(Wood et al., 1990), 3(Chapter 3; Baker et al., 2009a), 4(Chapter 3; Brauner et al., 2004), 5(R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong, M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong,

and C.J.B., unpublished observations; reviewed in Shartau et al., 2016a), 6(Chapter 3; Wood and LeMoigne, 1991), 7(Larsen et al., 1997), 8(Chapter 3; Heisler, 1982), 9(Wright et al., 1988)]. Phylogenetic relationships are based on (2009) and branch lengths are taken from various references utilizing fossil and molecular estimates of divergence times (Aschliman et al., 2012; Betancur-R et al., 2013; Betancur-R et al., 2015; Blair, 2005; Macqueen and Johnston, 2014; Meredith et al., 2011; Zhang et al., 2013); the phylogenetic tree was created using Mesquite (Maddison and Maddison, 2017).

Table 3.1: Plasma Cl^- and osmolarity, and hematocrit of fishes subjected to hypercarbia exposure.

Species	PCO_2 (kPa)	Exposure time (h)	Cl^- (mM)		Osmolarity (mM)		Hematocrit (%)	
			Control	CO_2	Control	CO_2	Control	CO_2
Polyodon spathula	1.5	3	111 ±4	113 ±3	248 ±6	252 ±5	27 ±2	18 ±2*
Acipenser transmontanus[1,2]	1.5	6	121 ±1	114 ±2*	265 ±3	266 ±5*		
	6	6	119 ±3	95 ±3*			31 ±3	33 ±3
	12	6	119 ±3	88 ±2*			31 ±3	24 ±2*
Atractosteus spatula	6	3	128 ±5	127 ±3	286 ±9	305 ±12	33 ±3	27 ±2
Lepisosteus oculatus	6	3	107 ±9	108 ±1	266 ±14	283 ±7	33 ±8	38 ±8
Colossoma macropomum	4	3	147 ±4	138 ±6			28 ±1	31 ±2
	20	3					22 ±1	24 ±2
Brycon amazonicus	4	3	119 ±4	121 ±6			42 ±1	35 ±4
Hoplosternum littorale	4	3	120 ±4	128 ±4			31 ±1	33 ±3
Pterygoplichthys pardalis[3]	4.3	6	111 ±5	108 ±3	247 ±7	246 ±5	40 ±2	40 ±2
Ictalurus punctatus	6	3	120 ±4	112 ±2	276 ±7	276 ±6	23 ±2	28 ±4
Oncorhynchus mykiss	1.5	3					27 ±2	35 ±2*
	3	3					27 ±2	51 ±4*
Oreochromis niloticus	6	3					21 ±2	28 ±8
Astronotus ocellatus	4	3	147 ±9	138 ±5				
Freshwater teleost[4]			125-132		262-274			

Significant differences between control and CO_2 exposures are indicated by asterisk ($P<0.05$). Typical freshwater teleost Cl^- and osmolarity values are shown for reference at the bottom of the table. 1(Baker et al., 2009a), 2(Baker and Brauner, 2012), 3(Brauner et al., 2004), 4(Edwards and Marshall, 2013).

Table 3.2: CO_2 tolerance assay in various fish species. CO_2 tension was increased at a rate of 2 kPa per hour, starting at normocarbia (~0.04 kPa PCO_2) until fish reached loss of equilibrium (LOE). Where LOE was reached, the CO_2 tension and time at which LOE was first recorded is indicated, and the max PCO_2 exposure fish were able to tolerate (within range of our equipment) is noted, and finally, the median and mean CO_2 tension that LOE occurred are indicated. The exposure was repeated in a couple of species due to sufficient number of animals. There were a couple of species where CO_2 exposure ended once the first animal reached LOE as that endpoint was deemed sufficient to determine the presence of preferential pH_i regulation (e.g. *Lepisosteus oculatus*). CO_2 tolerance in a few species was high as they tolerated CO_2 tensions higher than our equipment could measure (26.6 kPa PCO_2) and thus we used that CO_2 tension as an endpoint instead of LOE (e.g. *Colossoma macropomum*).

Species	n	# fish reaching LOE	Time to first LOE (h)	PCO_2 range of LOE (PCO_2 at 1st LOE – PCO_2 at last LOE)	Median PCO_2 at LOE	Mean PCO_2 at LOE
Entosphenus tridentatus	10	10	6.6	13.2 – 17.1	14.7	**14.6±0.4**
Potamotrygon spp.	10	10	5.0	10.1 – 11.9	11.0	**11.1±0.2**
Polyodon spathula	7	7	1.0	1.9 – 5.9	2.0	**2.7±0.5**
	8	8	0.6	1.1 – 1.9	1.4	**1.4±0.1**
Acipenser transmontanus	9	9	2.7	5.3 – 26	11.5	**14.6±2.3**
Lepisosteus oculatus	6	1	6.1	12.1 – n/a	n/a	**n/a**
	6	1	6.0	11.9 – n/a	n/a	**n/a**
Atractosteus spatula	8	1	6.0	12 – n/a	n/a	**n/a**
Arapaima gigas	10	8	9.7	19.3 – 26.7	26.7	**24.4±1.0**
Colossoma macropomum	10	0	>13.4	n/a (>26.7)	n/a	**n/a**
Electrophorus electricus	2	0	>13.4	n/a (>26.7)	n/a	**n/a**
Ictalurus punctatus	9	1	4.3	8.5 – n/a	n/a	**n/a**
	10	10	4.1	8.1 – 8.7	8.5	**8.4±0.1**

Species	n	# fish reaching LOE	Time to first LOE (h)	PCO_2 range of LOE (PCO_2 at 1st LOE – PCO_2 at last LOE)	Median PCO_2 at LOE	Mean PCO_2 at LOE
Ictalurus punctatus X Ictalurus furcatus	10	10	3.2	6.3 – 8.4	7.5	**7.4±0.2**
Pterygoplichthys pardalis	10	10	5.4	10.7 – 18	13.2	**14.0±0.9**
Oncorhynchus mykiss	9	9	1.8	3.5 – 5.7	5.1	**4.8±0.3**
Oncorhynchus kisutch	10	10	1.4	2.8 – 8.8	5.2	**5.5±0.7**
Synbranchus marmoratus	10	0	>13.4	n/a (>26.7)	n/a	**n/a**
Astronotus ocellatus	8	8	5.8	11.5 – 16.5	13.9	**13.9±0.6**
Oreochromis niloticus X *mossambicus* X *hornorum*	10	10	5.1	10.1 – 15.1	12.5	**12.6±0.5**
Lepidosiren paradoxa	8	0	>13.4	n/a (>26.7)	n/a	**n/a**

Chapter 4: Embryonic Common Snapping Turtles (*Chelydra serpentina*) Preferentially Regulate Intracellular Tissue pH During Acid-Base Challenges

4.1 Introduction

The nests of many reptiles naturally experience changes in carbon dioxide (CO_2) levels, often resulting in an elevated CO_2 (hypercarbia) rearing environment for the embryos. These conditions arise due to a number of biotic and abiotic factors including nest saturation from precipitation, metabolic activity of microorganisms, and from changes in embryonic metabolism (Ackerman, 1977; Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984). In nests of the broad-shelled river turtle (*Chelodina expansa*), green turtle (*Chelonia mydas*), and loggerhead turtle (*Caretta caretta*) CO_2 values can reach up to 5-8 kPa PCO_2 (Booth, 1998; Prange and Ackerman, 1974); similar PCO_2 tensions have been recorded in crocodilian nests (Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984).

The degree of disturbance and recovery from an acute hypercarbic-induced respiratory acidosis has been well described in adult amniotes, and initially it is typically characterized by reductions in both blood [extracellular pH (pH_e)] and tissue pH [intracellular pH (pH_i)] that change in a qualitatively similar manner. Compensation of pH_i is usually more rapid that of pH_e, but compensation in both compartments is coupled (Busk et al., 1997; Nestler, 1990; Siesjö et al., 1972; Wasser et al., 1991), which we define here as coupled pH regulation. This pattern of coupled pH_i and pH_e compensation following a respiratory acidosis is thought to be representative of vertebrates in general. However, in CO_2 tolerant fishes, it is becoming increasingly clear that pH_i in a number of species is tightly regulated in the complete absence of pH_e regulation (Baker et al., 2009a; Brauner and Baker, 2009; Brauner et al., 2004; Harter et al., 2014; Heisler, 1982; Shartau and Brauner, 2014), termed preferential pH_i regulation. Preferential pH_i regulation confers exceptional CO_2 tolerance by allowing animals to withstand severe

challenges to acid-base regulation (Brauner and Baker, 2009; Shartau and Brauner, 2014).

Chicken embryos between 60 and 90% of incubation subjected to hypercarbia (5 kPa PCO_2) for 24 h experienced a reduction in pH_e that was largely uncompensated (Burggren et al., 2012). Embryonic chickens are exceptionally hypercarbic tolerant as they can survive 1 h exposure to PCO_2 of 10 kPa where pH_e is reduced by ~0.8 pH units (Andrewartha et al., 2014), a degree of pH_e depression typically observed in animals that preferentially regulate pH_i (Shartau and Brauner, 2014). Amniotic embryos are enclosed within structures (e.g. eggshell, chorioallantoic membrane) that create diffusion barriers and limit or eliminate the ability for net acid excretion with the environment necessary for pH compensation. Thus, tolerance of a respiratory acidosis may be associated with preferential pH_i regulation, a phenomenon that has not been investigated previously in embryonic amniotes.

Embryonic turtles can survive chronic high CO_2 in both nest (see above) and incubation environments (Wearing et al., 2014), suggesting a high degree of CO_2 tolerance for chronic, and likely acute, CO_2 exposure. We were interested in how turtles respond to severe acute respiratory acid-base disturbances as the ability to tolerate high CO_2 could be associated with the capacity for preferential pH_i regulation, as observed in a number of fishes and a salamander during acute hypercarbia (Brauner and Baker, 2009; Shartau and Brauner, 2014), but never in amniotes. We hypothesized that embryonic turtles preferentially regulate pH_i allowing them to tolerate severe acute acid-base challenges. To test this hypothesis, we conducted two series of experiments. Series 1 investigated the pattern of acid-base regulation in normocarbia/normoxia-reared animals subjected to an acute respiratory acidosis at three developmental stages (70 and 90% of incubation, and yearlings) to assess the pattern of acid-base regulation during development. Next, in Series 2, we were interested if the pattern of acid-base regulation differed in embryos (at 90% of incubation) that had been reared under constant hypercarbia (representative of typical CO_2 tensions in a natural nest environment) and then exposed to a more severe acute respiratory acidosis or to an acute respiratory alkalosis. The acid-base status of turtles was assessed in the blood compartment by measuring pH_e, and in the tissues by measuring pH_i of heart, brain, liver, white muscle,

kidney, and lung. The results of this study indicate that embryonic turtles preferentially regulate pH_i, while the capacity for preferential pH_i regulation is reduced in yearlings as the transition to coupled pH regulation occurs.

4.2 Methods

4.2.1 Turtle embryo acquisition and incubation

Common snapping turtle eggs (*Chelydra serpentina* (Linnaeus, 1758)) were collected in north-western Minnesota, USA and transported by automobile to the laboratory at the University of North Texas (Minnesota Department of Natural Resources Permit No. 19772 to DAC). Eggs were staged to determine approximate age of each clutch (53-55 d total incubation period at 30°C (Yntema, 1968)); a clutch being embryos from the same nest. Eggs were incubated at 30°C in a walk-in, constant temperature room on a 14h:10h light:dark photoperiod. All embryos were incubated in plastic containers, placed in a bed of moist vermiculite mixed in a 1:1 ratio of vermiculite:water. Water content of the vermiculite was maintained by weighing the box twice weekly and adding water as needed to keep the mass constant.

Embryos from each clutch were divided into two groups, and reared in normocarbic/normoxic (0.03 kPa PCO_2, 21 kPa PO_2; "NC") or hypercarbic/normoxic (3.5 kPa PCO_2, 21 kPa PO_2; "HC3.5") conditions from that point onward. Exposure began at ~18-22% of incubation (10-12 days post-laying, where 100% of incubation would correspond with hatch), determined by dissection of at least two representative embryos from each clutch as described previously (Crossley and Altimiras, 2005; Eme et al., 2011). For NC incubation, embryos were sealed inside large Ziplock bags, with two holes in the bag that allowed parallel inflow and outflow of gas in normoxic/normocapnic conditions in a walk-in Percival® incubator (Percival Scientific, Perry, IA). HC3.5 embryos were incubated in separate 0.3 m^3 Percival incubators (model I30NLX, Percival Scientific, Perry, IA) fitted with IntellusUltra™ controllers and an IntellusUltra™ Web Server that allowed CO_2 to be regulated ±0.2% and for O_2 and CO_2 levels to be monitored remotely. The target gas tensions (3.5 kPa PCO_2, 21 kPa PO_2) were achieved

using rotameters and Intellus™ solenoid controllers, which controlled the upstream supply of compressed O_2 and CO_2, respectively. Incoming O_2 and CO_2 levels were monitored with analyzers (S-A/I and CD-3A, respectively; Ametek Applied Electrochemistry, IL, USA) connected to a PowerLab® with LabChart Pro® software (v 7 ADInstruments, CO, USA).

Yearlings from the previous clutch year (2013) were kept in 70 l tanks at 28°C with sufficient water for voluntary submergence and access to room air. They were fed 3 times weekly, and animals were fasted for 5 days prior to experimentation.

Measurements were made in embryos at 70% (N=8) and 90% (N=8) of incubation, which reflected developmental stages 22/23 and 25/26, respectively, or in yearlings (N=6) that were approximately one year old. This study used embryos from 13 clutches; each experimental exposure used typically one embryo, and occasionally two, per clutch. Three clutches of yearlings were used, two animals per clutch for each experimental exposure. All studies were approved by UNT IACUC #11-007.

4.2.2 Experimental protocols

Embryos: Surgical procedures and experimental set-up

Embryos were removed from their respective incubation chambers and candled to identify a tertiary chorioallantoic membrane (CAM) artery. Embryos were placed in a temperature-controlled surgical chamber (30°C) under normocarbic/normoxic (NC) conditions and ~1 cm^2 of the eggshell was removed under a dissection microscope (Leica MZ6 or MZ3; Leica Microsystems, Waukegan, IL, USA). A tertiary CAM artery was isolated for arterial pressure monitoring and blood sampling in the experimental series described below. An occlusive catheter was inserted into a tertiary CAM using heat-pulled, heparinized, and saline-filled PE-50 tubing, as previously described (Crossley and Altimiras, 2005; Crossley and Altimiras, 2000). The surgical preparations were minimally invasive and no anesthesia/analgesia was used; the entire surgical procedure took 7-10 min. Following catheterization, the catheter was fixed to the shell with cyanoacrylic glue and embryos were placed in a water jacketed multi-chamber experimental unit (~700 cm^3 per chamber, one embryo per chamber, placed on cotton)

and allowed to acclimate for at least 60 min prior to experimentation (described below) at incubation gas tensions.

Temperature in the chambers was maintained at 30^{o}C by recirculating water from a constant temperature circulator (VWR International, LLC, West Chester, PA, USA). Each chamber consisted of a container fitted with a lid with three ports that allowed the catheter and airlines to enter the chamber. To prevent changes in chamber temperature due to incoming gas flow, all incoming gas traversed a 1 m copper line submerged within the constant temperature circulator's water bath. Gas was forced into each chamber at a flow rate of 200 ml min^{-1}. Cardiovascular measurements of blood pressure and heart rate were obtained by connecting the arterial catheter with saline-filled PE50 tubing to a pressure transducer held 1-3 cm above the egg, connected to an amplifier, and the pressure signal acquired at 40 Hz using PowerLab data recording system (ADInstruments, CO, USA) connected to a computer running Chartpro software (v 7.4 ADInstruments). Pressure transducers were calibrated prior to each measurement period with a vertical column of saline, and heart rate was determined with a software tachograph that integrated the arterial pressure trace. Cardiovascular measurements were made to verify embryos were alive during these acid-base exposures and to avoid sampling unhealthy animals, as well as to quantify cardiovascular changes during acid-base challenges.

Yearlings: Experimental set-up

Yearling turtles were placed in a water-jacketed, multi-chamber, stainless steel experimental apparatus (~4000 cm^3 per chamber, one animal per chamber) containing ~1000 ml tap water and allowed to acclimate for at least 90 min prior to experiments (described below). Temperature in the chambers was maintained at 30^{o}C by recirculating water within the water jacket from a constant temperature circulator (VWR International, West Chester, PA, USA). Each chamber consisted of a container fitted with a lid with small holes that allowed air lines to enter the chamber. Air or $N_2/O_2/CO_2$ gas mix was bubbled into the water using an air stone to ensure sufficient gas flow.

4.2.3 Experimental treatments

Series 1: Acid-base status during development in normocarbic normoxia following exposure to severe hypercarbic hypoxia.

The specific objective of this series was to induce a severe respiratory acidosis and investigate for the presence or absence of preferential pH_i regulation rather than mimicking the natural rearing environment of the turtle. NC reared animals that had been placed in individual chambers as described above were sampled (as described below) at either 70% of incubation or 90% of incubation, or as yearlings after exposure to 1 h of NC (control) or 1 h exposure to severe hypercarbic hypoxia (13 kPa PCO_2 and 9 kPa PO_2; HC13). The 1 h exposure time was chosen because in fish preferential pH_i regulation is observed at maximal pH_e depression, which occurs within 1 h of hypercarbia exposure (Baker, 2010; Baker et al., 2009a); no comparable embryonic or reptile studies exist to provide guidance for exposure times (Everaert et al., 2011). HC13 was generated using three mass flow controllers (GFC Aalborg; Orangeburg, NY, USA) and command module (Model SDPROC, Aalborg; Orangeburg, NY, USA) supplied with compressed O_2, CO_2, and N_2 to achieve the desired gas mix. O_2 and CO_2 levels were monitored with analyzers (S-A/I and CD-3A, respectively; Ametek Applied Electrochemistry, IL, USA). Gas composition in the chamber changed within 1-2 min and was maintained for the remaining hour prior to sampling.

Series 2: Response to a respiratory acidosis or respiratory alkalosis at 90% of incubation in embryos reared under constant hypercarbia levels.

Embryos reared in HC3.5 at 90% of incubation were sampled to examine the effect of hypercarbic rearing on acid-base balance at CO_2 tensions likely representative of the natural nest environment. Next, the effect of respiratory acidosis on HC3.5 reared embryos was examined by exposing HC3.5 embryos at 90% of incubation to HC13 for 1 h and then sampled as described below. To examine the effect of a respiratory alkalosis, HC3.5 reared embryos were exposed to normocarbic normoxia for either 3 or 24 h and then sampled as below.

Due to limited numbers of HC3.5 reared embryos in Series 2, only embryos at 90% of incubation were investigated. We chose this developmental stage over 70% of incubation because we felt they would be more likely to tolerate the severe acid-base challenges and increase the likelihood of Series 2 being successful. There were no turtles continuously reared to yearlings under HC3.5, thus, we could not include yearlings in Series 2.

4.2.4 Blood sampling, animal euthanasia, tissue sampling and ions

- Embryos

Embryonic heart rate and blood pressure were continuously recorded prior to sampling. Following a 1 h exposure period approximately 70-200 µl of blood was sampled from the cannulated CAM artery by disconnecting the cannula from the pressure transducer and allowing the blood to passively flow into a 1ml heparinized plastic syringe; blood pH (pH_e) and total CO_2 (TCO_2) were measured immediately. pH_e was measured using a thermostated capillary pH electrode (model BMS 3 MK 2; Radiometer; Copenhagen, Denmark) that was calibrated daily with buffer solutions (BDH5050, pH 7.38 and BDH5058, pH 6.86; VWR; Radnor, PA, USA). TCO_2 was measured using a total CO_2 analyzer (Corning model 965 Analyzer; Essex, United Kingdom) and was calibrated using freshly prepared 0, 10, and 25 mmol l^{-1} $NaHCO_3$. Embryos were then euthanized with an overdose of sodium pentobarbital (100mgkg^{-1}) injected into the CAM artery. Tissues (heart, brain, liver, white muscle, kidney, and lung) were then quickly dissected (within 5 min), placed in micro-centrifuge tubes, frozen in liquid nitrogen and stored at -80^{o}C for later measurements of pH_i. Tissue was later ground under liquid nitrogen and pH_i was measured using the metabolic inhibitor tissue homogenate method (MITH: see Appendix for detailed description of this method); this technique has been validated (Baker et al., 2009b; Portner et al., 1990) and used in fish (Baker and Brauner, 2012; Baker et al., 2015; Brauner et al., 2004; Regan et al., 2016) and non-fish (Busk et al., 1997; Galli and Richards, 2012) studies. Plasma Na^+, K^+, Cl^-, and Ca^{2+} were measured in embryos at 90% of incubation at each rearing condition using Nova Biomedical Stat profile prime (Waltham, MA, USA).

– *Yearlings*

To sample blood and tissues in yearlings, turtles were removed from the chamber, euthanized with an overdose of isoflurane and the plastron removed and the heart exposed. Blood was sampled (~200-300 µl) from the right aorta using a 1 ml syringe with a 30 gauge heparinized needle. Tissues (heart, brain, liver, white muscle, kidney, and lung) were immediately dissected out (within 6-7 min) and frozen for later analysis as described above. Due to the greater blood volume collected in yearlings, blood PCO_2 was measured at the same time as pH_e using a PCO_2 electrode (E201/E5037; Loligo Systems; Denmark) thermostated at 30°C in a Radiometer BMS 3 MK 2 (Copenhagen, Denmark) calibrated daily with humidified pre-mixed gases. All measurements of pH_i and pH_e, and TCO_2 were measured as described above.

4.2.5 Calculations and statistical analyses

Plasma [HCO_3^-] and PCO_2 were calculated using measured TCO_2 and pH values as described by Brauner et al. (2004). The CO_2 solubility coefficient and pKa were calculated using equations from Heisler (1984) which were adapted, and experimentally validated, for use with reptile blood (Stabenau and Heming, 1993). To determine how a 1 h HC13 exposure changes [H^+] relative to NC (control) [H^+], pH_i values were converted to [H^+] ([H^+]=10^{-pH}) and HC13 [H^+] was subtracted from NC [H^+] to calculate the net [H^+] difference. This was done for each tissue at each developmental age and was plotted as mean ± s.e.m.

All data was analyzed using R version 3.1.0 (The R Foundation for Statistical Computing). Homogeneity of variances was tested with the Levene's test (P<0.05) and normality of distributions was tested with the Shapiro-Wilkinson test (P<0.05). Differences between control and treatment group means of individual measurements were compared using a Welch two-sample t-test (P<0.05). Comparisons of means across treatments, tissues and/or developmental age were conducted using either a one-way or two-way ANOVA (Tukey post hoc, P<0.05) as appropriate. Data that did not meet the assumption of normality for a one-way ANOVA were analyzed using the Kruskal-Wallis test (P<0.05). Absolute blood pressure was corrected for the pressure transducer's distance above the egg. Mean arterial pressure (kPa) and mean heart rate (beats min^{-1})

were calculated from the individual mean values for embryos in each exposure group. Mean arterial pressure and mean heart rate for individual embryos were based on stable period at 10 min intervals over the exposure time period. Mean arterial pressure and mean heart rate during exposure were compared to unexposed measurements using a one-way ANOVA, followed by a Tukey post hoc (P<0.05). All values are presented as mean±s.e.m; sample size for NC embryos are N=8, NC yearlings are N=6, and HC3.5 embryos are N=6. All figures were created using GraphPad Prism *v*5.0 (GraphPad Software Inc., 2007).

4.3 Results

4.3.1 Series 1: Acid-base status during development in normocarbic normoxia following exposure to severe acute hypercarbic hypoxia

Animals reared at NC and transferred to HC13 for 1 h exhibited a significant reduction in pH_e and a significant increase in blood PCO_2 at all three developmental ages (Welch 2-sample t-test, P<0.05) (Fig. 4.1A) as expected *a priori*. Blood [HCO_3^-] did not change significantly (Fig. 4.1A). The pattern of changes in pH_i, however, differed between ages. At 70% of incubation, hypercarbia was associated with a significant increase in pH_i of the brain, white muscle, and lung but no statistically significant change was observed in heart, liver, or kidney (Fig. 4.1B); at 90% of incubation only heart pH_i significantly increased while no changes in liver, brain, white muscle, lung, or kidney were observed (Fig. 4.1C). In yearlings there were no significant changes in pH_i of any tissues (Welch 2-sample t-test, P>0.05), however, there was a trend toward a reduction in pH_i in most tissues (Fig. 4.1D).

To assess the effect of development and tissue type on acid-base changes following acute hypercarbia, [H^+] was calculated from pH_i, then tissue [H^+] following 1 h hypercarbia was subtracted from the respective NC (control) tissue [H^+] for each tissue type and at each developmental age. There was a significant effect of developmental age on the difference in tissue [H^+] from control, where a progressive statistically significant increase in tissue [H^+] was observed with an increase in developmental age (two-way

ANOVA, Tukey's post hoc; $P<0.01$) indicating a progressive reduction in the ability to preferentially regulate pH_i. Additionally, the various tissues respond differently as development proceeds as the interaction of developmental age and tissue significantly affected the net change in tissue $[H^+]$ (i.e. the changes between treatment and control $[H^+]$ between tissue differ significantly when developmental age is considered) (two-way ANOVA, $P<0.05$) (Fig. 4.2).

Cardiovascular measurements indicated that embryos at 70% of incubation reared in NC and exposed to HC13 exhibited no significant changes in blood pressure (0.50 ± 0.08 kPa) or heart rate (48.3 ± 9.1 beats min^{-1}) from controls (one-way ANOVA, $P>0.05$). In embryos at 90% of incubation, blood pressure and heart rate were reduced during HC13 exposure from 1.14 ± 0.09 kPa to 0.82 ± 0.06 kPa and 53.2 ± 4.6 beats min^{-1} to 36.7 ± 2.7 beats min^{-1}, respectively (one-way ANOVA, Tukey's post hoc, $P<0.001$).

4.3.2 Series 2: Response to an acute respiratory acidosis or alkalosis at 90% of incubation in embryos reared under constant hypercarbia

Embryos at 90% of incubation reared at HC3.5 had increased pH_e, blood PCO_2 and $[HCO_3^-]$ compared to those reared in NC (Fig. 4.3A-C). pH_i was also significantly elevated in all tissues, except liver (Fig. 4.3D-I). Exposure of HC3.5 reared embryos at 90% of incubation to HC13 for 1 h resulted in a significant reduction in pH_e and a significant increase in blood PCO_2 but no change in blood $[HCO_3^-]$ (Welch 2-sample t-test, $P<0.001$) (Fig. 4.4A). Heart pH_i was significantly reduced; other tissues did not change (Welch 2 sample t-test, $P<0.05$) (Fig. 4.4B). Plasma ions (Na^+, K^+, Cl^-, and Ca^{2+}) were measured in untreated embryos at 90% of incubation to assess for differences due to rearing conditions that may affect acid-base status between the groups. The HC3.5 reared embryos had a greater $[K^+]$ compared to the NC reared embryos (t-test, $P<0.05$). There were no differences in other ion concentrations (Table 4.1).

Embryos at 90% of incubation reared in HC3.5 and transferred to NC for 3 or 24 h exhibited a significant increase in pH_e (one-way ANOVA, $P<0.0001$) and reduction in blood PCO_2 (one-way ANOVA, Tukey's post hoc, $P<0.001$) (Fig. 4.5A). There was a significant reduction in $[HCO_3^-]$ following 24 h NC exposure (one-way ANOVA, Tukey's post hoc, $P<0.01$) (Fig. 4.5A). Tissue pH_i was unchanged at 3 h but at 24 h, heart

and brain pH_i were significantly reduced (one-way ANOVA, Tukey's post hoc, $P<0.05$) (Fig. 4.5B,C). Cardiovascular measurements showed that embryos at 90% of incubation reared at HC3.5 had reductions in blood pressure and heart rate during HC13 exposure from 0.96 ± 0.05 kPa to 0.67 ± 0.04 kPa and 58.1 ± 1.3 beats min^{-1} to 39.6 ± 1.5 beats min^{-1}, respectively (one-way ANOVA, Tukey's post hoc, $P<0.001$).

4.4 Discussion

Preferential pH_i regulation has been documented in a number of fishes, and in an aquatic salamander, but never before in amniotes (Cameron, 1989a; Everaert et al., 2011; Shartau and Brauner, 2014). We hypothesized that embryonic turtles preferentially regulate pH_i during a severe acute acidosis, which is supported by our findings here on snapping turtles; this is the first time this pattern of pH regulation has been identified in an amniote. These results suggest that coupled pH regulation is not the strategy used during embryonic development of snapping turtles and demonstrates that preferential pH_i regulation is likely important for tolerating acute respiratory acid-base disturbances in this amniote species at this development stage.

4.4.1 Capacity for preferential pH_i regulation shifts during development

Exposure of NC reared turtles to HC13 greatly increased blood PCO_2 (Fig. 4.1A); the difference between blood and environmental PCO_2 of 13 kPa likely represents non-equilibrium between the animals and the environment due to the short exposure time. Despite the lack of complete CO_2 equilibration, turtles experience large reductions in pH_e (which was the objective of the treatment) but there was no reduction in pH_i (Fig. 4.1) consistent with preferential pH_i regulation. However, there appears to be a reduction in the capacity for pH_i regulation between the younger embryos and yearlings. During 1 h HC13 exposure, three tissues exhibited a significant increase in pH_i in embryos at 70% of incubation, while this was observed in only one tissue in 90% of incubation embryos and none in yearlings (Fig. 4.1B-D), suggesting younger embryos possess a greater capacity for preferential pH_i regulation. When contrasted to adult western painted turtles, the lack

of pH_i change during hypercarbia in embryos is impressive as adult western painted turtles (the only known study to measure pH_e and pH_i in adult turtles exposed to hypercapnia) (Wasser et al., 1991) experiencing 1 h of hypercapnia exhibited severe reductions in pH_e, and pH_i of heart, liver, brain, and skeletal muscle. The difference between pH of hypercapnic exposed and control animals is plotted for blood and tissues (Wasser et al., 1991) in Figure 4.6, along with relevant results from this study to highlight the large pH_i reductions in adult turtles compared to embryos.

The differences in the pattern of acid-base regulation between snapping turtle embryos and yearlings, and western painted turtle adults is likely due to changes in the capacity for preferential pH_i regulation and buffering capacity. An increase in pH_i from control values during an acidosis (or decrease during an alkalosis) is due to preferential pH_i regulation and not buffer capacity, as the latter can only delay or minimize the reductions in pH during an acidosis (or increases during an alkalosis). Turtles appear to transition from preferentially regulating pH_i to having coupled pH regulation.

4.4.2 Rearing condition alters blood and tissue acid-base status

Rearing condition appears to affect blood and tissue acid-base status. Embryos at 90% of incubation reared at HC3.5 had a blood PCO_2 of 3.6 kPa PCO_2 (Fig. 4.3B), which was slightly higher than incubation PCO_2 of 3.5 kPa PCO_2. This indicates that these embryos were in equilibrium with environmental PCO_2, as would be expected, and the slightly higher blood PCO_2 would permit the release of metabolically produced CO_2 to their environment. Additionally, these embryos experienced a higher pH_e and blood $[HCO_3^-]$ compared to NC reared embryos (Fig. 4.3A,C) suggesting these embryos have compensated pH_e in chronic hypercarbia; pH_i was also elevated in all tissues, except liver (Fig. 4.3D-I). The increase in blood HCO_3^- (Fig. 4.3C) and plasma K^+ (Table 4.1) may indicate that these embryos compensate pH_e similar to chicken embryos during chronic elevations in CO_2, as the latter control pH_e by a combination of HCO_3^- uptake from the shell and excretion of H^+ into albumen in exchange for K^+ (Bruggeman et al., 2007; Crooks and Simkiss, 1974; Rowlett and Simkiss, 1989). The increase in blood HCO_3^- may facilitate pH_i regulation in turtle embryos by providing a greater HCO_3^- gradient of HCO_3^-/Cl^- exchange.

4.4.3 Acid-base regulation during development

Changes in the pattern of pH_i regulation during development are expected as a single cell develops into a complex organism. In the earliest developmental stages, cells cannot rely on extracellular pH regulation as the extracellular compartment does not yet exist; appropriately, *in vitro* studies measuring pH_i of post-fertilization single celled oocytes of mammals have shown that they are capable of regulating and defending pH_i against external acid-base challenges (Erdogan et al., 2005; FitzHarris and Baltz, 2009; Lane, 1999; Squirrell et al., 2001). Similarly, Molich and Heisler (Molich and Heisler, 2005) found that early stage embryonic cells of zebrafish (*Danio rerio*) regulate pH_i when exposed to changes in ambient PCO_2. Aside from studies on pH_e regulation in chicken embryos, which show incomplete pH_e regulation and are suggestive of preferential pH_i regulation, there are no other studies, to our knowledge, investigating acid-base regulation in embryonic amniotes or vertebrates once the extracellular space and circulatory system develops (Brauner, 2008; Everaert et al., 2011). Recently, however, the authors investigated the response of American alligator embryos to severe respiratory acidosis and found that they also preferentially regulate pH_i, similar to turtle embryos shown here (Shartau et al., in press).

During ontogeny, the capacity for coupled pH regulation increases due to the development of the extracellular space and necessary structures (e.g. cardiovascular, respiratory, and renal systems). Preferential pH_i regulation has not been identified in adult amniotes as pH_i is coupled to changes in pH_e during acid-base disturbances (Baldwin et al., 1995; Malan et al., 1985; Nestler, 1990; Siesjö et al., 1972; Wasser et al., 1991; Wood and Schaefer, 1978); however, this is not the case in all adult vertebrates. A number of fishes (Brauner and Baker, 2009; Shartau and Brauner, 2014), including a salamander (Heisler et al., 1982), preferentially regulate pH_i when subjected to severe acute acid-base disturbances despite reductions of $pH_e > 1$ pH units.

Snapping turtle embryos and yearlings are tolerant of acute hypercarbia, similar to other species capable of preferential pH_i regulation; this pattern of pH regulation appears to confer exceptional tolerance to CO_2 tensions up to 12 kPa PCO_2 (Baker et al., 2009a; Brauner and Baker, 2009; Shartau and Brauner, 2014). Without preferentially regulating

pH_i, it is unlikely these animals could tolerate, and thus, be able to maintain acid-base status during high CO_2 tensions due to putative limitations on pH_e regulation. The "bicarbonate concentration threshold", originally described by Heisler (Heisler, 1984; Heisler et al., 1982) limits plasma [HCO_3^-] uptake to approximately 27-33 mmol l^{-1} which limits complete pH_e compensation to CO_2 tensions below ~2-2.5 kPa PCO_2 (Brauner and Baker, 2009). In addition to conferring exceptional tolerance to hypercarbic-induced acidosis, preferential pH_i regulation appears to play a role in short-term pH_i regulation during both metabolic acidoses, metabolic alkalosis (Harter et al., 2014), and respiratory alkalosis (Fig. 4.5).

Similar to some fishes, including the armoured catfish (*Pterygoplichthys pardalis*), preferential pH_i regulation acts as a general pattern of acid-base regulation in turtle development as it protects against respiratory/metabolic acidosis of HC13 exposure (Fig. 4.1). Additionally, embryos reared at 3.5 kPa PCO_2, which likely mirror natural nest conditions, largely maintained pH_i during both HC13 and NC exposure, which create an acidosis and alkalosis, respectively (Fig. 4.4; 4.5); this suggests that preferential pH_i regulation is a pattern of acid-base regulation that is used during the course of development, conferring robust capacity to cope with acid-base challenges.

Cardiovascular function may be protected by preferential pH_i regulation

Preferential pH_i regulation may protect cardiac function in embryos at 70% of incubation. Blood pressure and heart rate did not change during severe acute acidosis, this response is similar to what is seen in white sturgeon (Baker et al., 2011) and armoured catfish (Hanson et al., 2009) during acute hypercarbia, both preferential pH_i regulators; however, cardiac function in embryos at 90% of incubation was not preserved. Difference in cardiac function between development ages may be due to the increased metabolic demand of older embryos being depressed by changes in CO_2 and O_2 (Erasmus et al., 1971), as in adult turtles cardiac function is reduced during lower metabolic demand (Jackson, 1987; Jackson et al., 1991).

4.4.4 Conclusions and perspectives

Preferential pH_i regulation has only been described a handful of times in fishes and amphibians (Baker et al., 2009a; Brauner and Baker, 2009; Brauner et al., 2004; Harter et al., 2014; Heisler, 1982; Heisler et al., 1982; Shartau and Brauner, 2014), but now our findings indicate that an amniote, the common snapping turtle, can also preferentially regulate pH_i. It is intriguing to think that preferential pH_i regulation may represent the "default" pattern of acid-base regulation used during development, starting from the single cell oocyte, and in some animals is maintained from this embryonic condition through to the adult stage. Clearly this is an area worthy of further investigation. Understanding the pattern of acid-base regulation in embryos and adults, and the transition between these different patterns of pH regulation will provide significant insight into acid-base homeostasis during development of amniotes, and vertebrates in general.

In conclusion, we demonstrated the first occurrence of preferential pH_i regulation in an amniote; furthermore, we also found that the capacity for preferential pH_i regulation changed during development between embryo to yearling. Preferential pH_i regulation in developing snapping turtles and other amniotes, such as American alligators (Shartau et al., in press), likely plays an important role in allowing embryos to successfully develop when faced with acute acid-base challenges for which typical adult mechanisms of acid-base compensation are unavailable. Future studies should investigate whether preferential pH_i regulation is used during development of other amniotes, and vertebrates; it would be interesting to assess if the capacity for pH_i regulation changes from embryo to adult in animals that are able to preferentially regulate pH_i as adults. Additionally, investigating the cellular and molecular mechanisms of preferential pH_i regulation, and how they change during development will be an important contribution to understanding acid-base physiology in vertebrates.

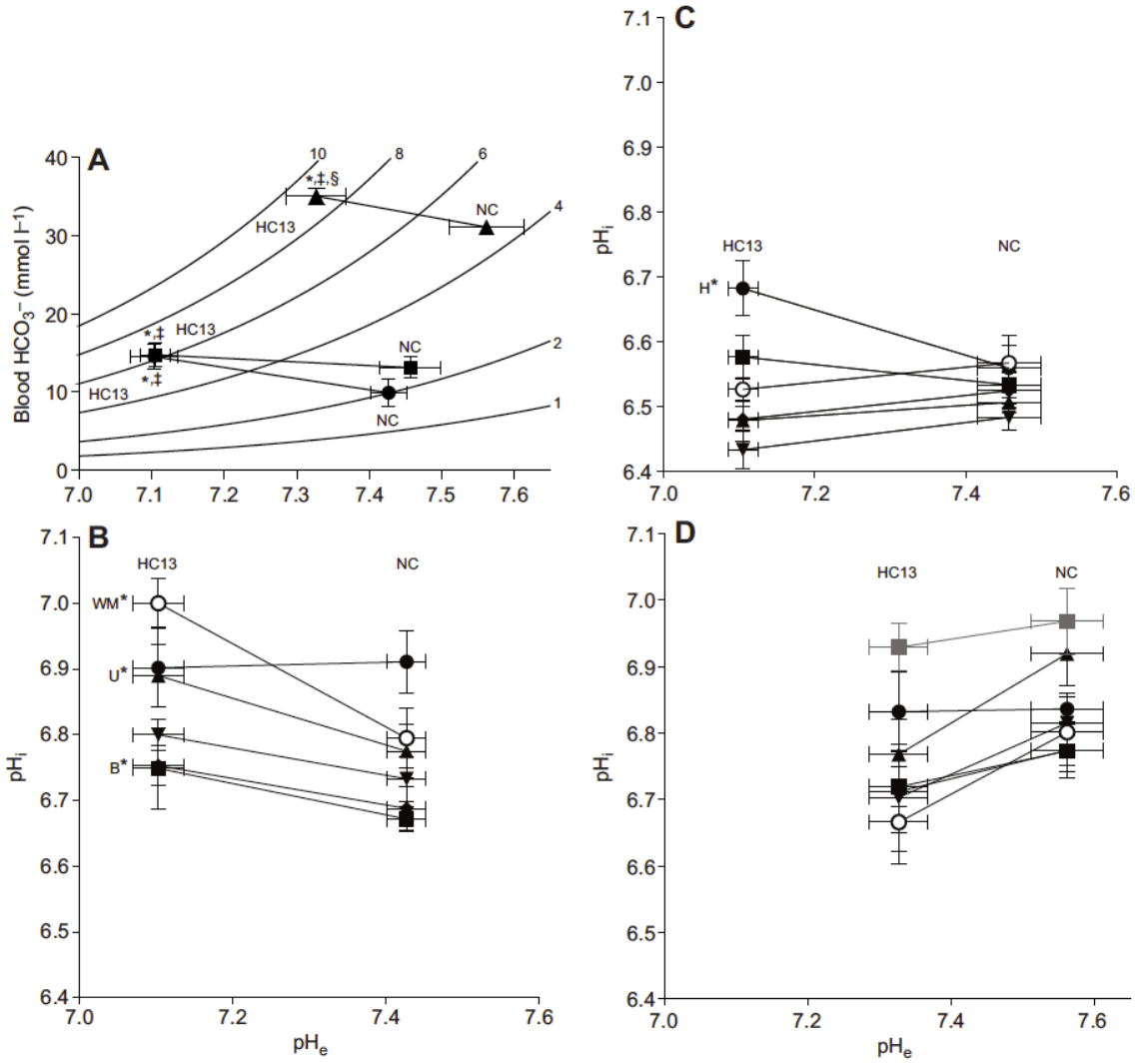

Figure 4.1: Effect of exposure to an acute respiratory acidosis in common snapping turtle (*Chelydra serpentina*) embryos (at 70 or 90% of incubation) or yearlings in Series 1 on blood and tissue acid-base status. Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH-HCO_3^- plot. Embryos at 70% of incubation (●), 90% of incubation (■), or yearlings (▲) were sampled in normocarbia (0.03 kPa PCO_2, 21 kPa PO_2; NC) or following 1 h hypercarbic hypoxia (13 kPa PCO_2, 9 kPa PO_2; HC13) exposure; curved lines represent PCO_2 isopleths (A). The relationship between pH_e and tissue pH (pH_i) in snapping turtles is indicated for 70% of incubation (B), 90% of incubation (C), and yearlings (D) following 1 h exposure to HC13. Tissues are indicated by the following symbols: heart (●, H), liver (■, L), lung (▲, U), kidney (▼, K), brain (◆, B), white muscle (○, WM) and red cell (■, RBC - yearlings only). Values are presented as means±s.e.m; n=8 for 70 and 90% of incubation, and n=6 for yearlings. A: symbols indicate significant differences (P<0.05) between

control (NC) and treatment (HC13) for pH_e (*), blood PCO_2 (∇), and blood HCO_3^- (Φ). B, C and D: *significant differences in pH_i from the NC group, letter next to asterisk indicates tissue ($P<0.05$).

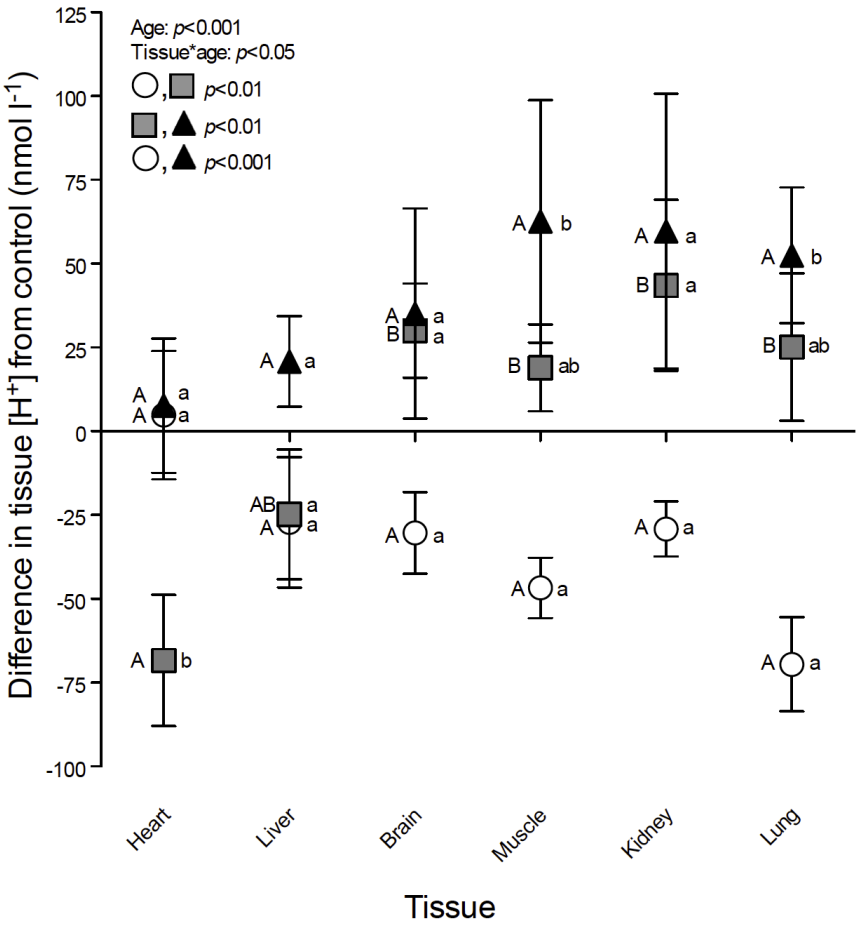

Figure 4.2: Difference in tissue [H^+] from control following 1h exposure to hypercarbia hypoxia (13 kPa PCO_2, 9 kPa PO_2; HC13) relative to normocarbic (0.03 kPa PCO_2, 21 kPa PO_2; NC) reared common snapping turtles (*Chelydra serpentina*) of Series 1. Concentrations of H^+ were calculated from tissue pH ($[H^+]=10^{-pH}$) and the mean NC [H^+] was subtracted from individual HC13 [H^+] values to calculate a mean difference [H^+] ± s.e.m. This was done for each tissue at each developmental age. 70% of incubation (○), 90% of incubation (■) and yearlings (▲). Positive [H^+] values indicate an increase in tissue [H^+] and negative [H^+] values indicate a reduction in tissue [H^+]. Significant differences between [H^+] changes across developmental ages and tissues were determined using a 2-way ANOVA, followed by Tukey's post hoc (n=8 for 70 and 90% of incubation, and n=6 for yearlings). Uppercase letters that differ indicate significant differences between tissues in the same developmental age and lowercase letters that differ indicate significant differences between developmental age in the same tissue following separate 1-way ANOVA followed by Tukey's post hoc (P<0.05).

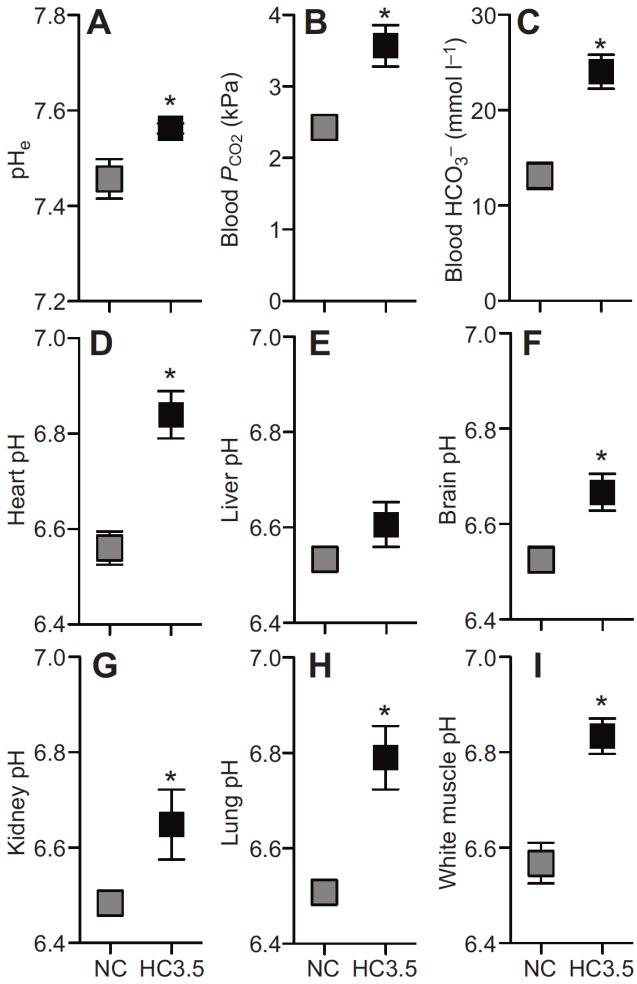

Figure 4.3: Changes in blood and tissue acid-base status in common snapping turtles (*Chelydra serpentina*) embryos at 90% of incubation continuously reared in either normocarbia or hypercarbia. (A) blood pH, (B) blood PCO_2 (kPa), (C) blood HCO_3^- (mmol l^{-1}), (D) heart pH, (E) liver pH, (F) brain pH, (G) kidney pH, (H) lung pH, and (I) white muscle pH, where different incubation conditions are indicated as follows: normocarbia (0.03 kPa PCO_2, 21 kPa PO_2; NC, ■) and hypercarbia (3.5 kPa PCO_2, 21 kPa PO_2; HC3.5, ■). Data are means ± s.e.m.; n=8 for NC embryos, and n=6 for HC3.5 embryos. These data are re-plotted from figures 1 and 4. Significant differences between rearing conditions are indicated by asterisk (P<0.05).

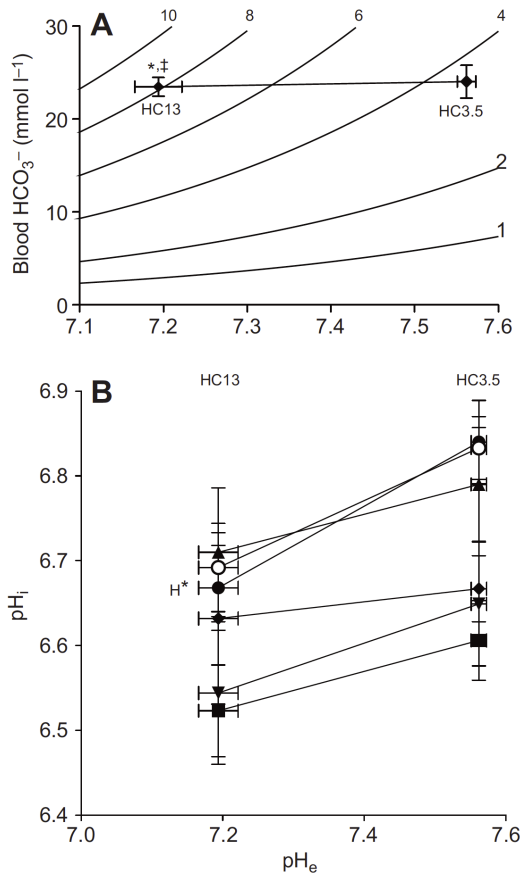

Figure 4.4: Effect of exposure to an acute respiratory acidosis in snapping turtle embryos (*Chelydra serpentina*) at 90% of incubation in Series 2 reared continuously and sampled in hypercarbia (3.5 kPa PCO_2, 21 kPa PO_2; HC3.5) or following 1 h exposure to hypercarbic hypoxia (13 kPa PCO_2, 9 kPa PO_2; HC13). Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH-HCO_3^- plot. Embryos were sampled in HC3.5 or following 1 h HC13 exposure; curved lines represent PCO_2 isopleths (A). The relationship between pH_e and tissue pH (pH_i) in snapping turtles following 1h exposure to HC13. Tissues are indicated by the following symbols: heart (●, H), liver (■, L), lung (▲, U), kidney (▼, K), brain (◆, B), and white muscle (○, WM). Values are presented as means ± s.e.m. (n=6). A: symbols indicate significant differences (P<0.05) between HC3.5 and HC13 for pH_e (*) and blood PCO_2 (▽). B: *significant differences in pH_i from the NC group, letter next to asterisk indicates tissue (P<0.05).

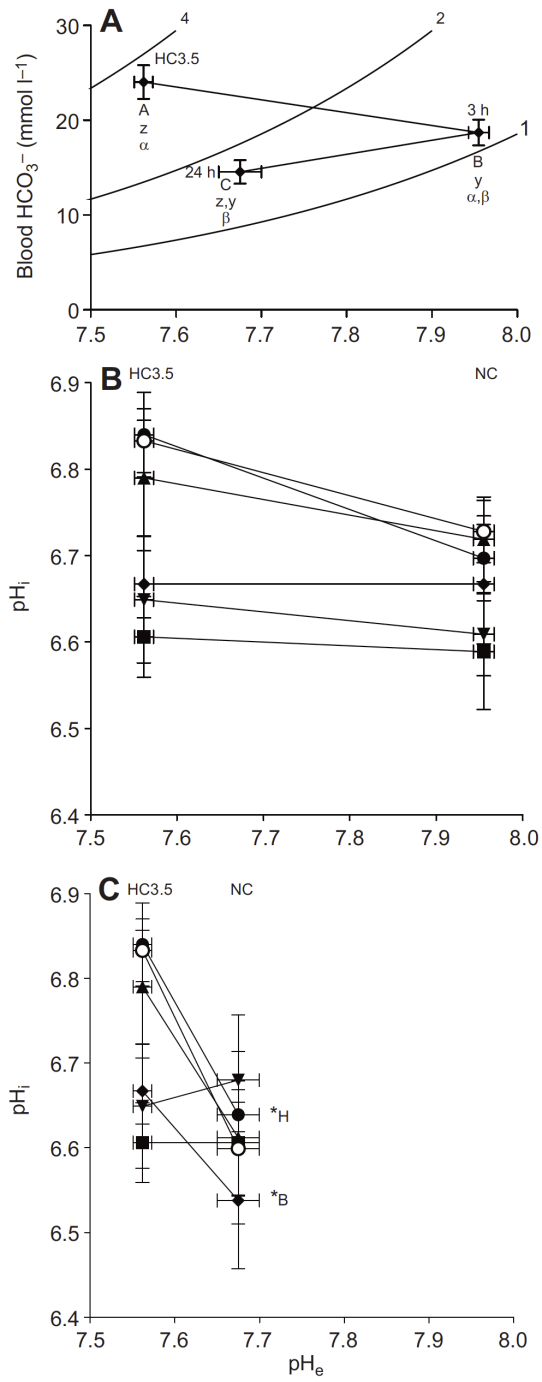

Figure 4.5: Effect of exposure to normocarbia in snapping turtle embryos (*Chelydra serpentina*) at 90% of incubation in Series 2 reared continuously and sampled in hypercarbia (3.5 kPa PCO_2, 21 kPa PO_2; HC3.5) or following 1 h exposure to normocarbia (0.03 kPa PCO_2, 21 kPa PO_2; NC) for either 3 or 24 h. Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH-HCO_3^- plot. Embryos were sampled in HC3.5 or following 1 h HC13 exposure; curved lines represent PCO_2 isopleths (A). The relationship between pH_e and tissue pH (pH_i) in snapping turtles following 1h

exposure to HC13 for 3 h (B) or 24 h (C). Tissues are indicated by the following symbols: heart (●, H), liver (■, L), lung (▲, U), kidney (▼, K), brain (◆, B), and white muscle (○, WM). A: different letters indicate significant differences (P<0.05) between control (NC) and treatment (HC13) for pH_e (Uppercase letters), blood PCO_2 (lowercase letters), and blood HCO_3^- (Greek letters). B, C and D: *significant differences in pH_i from the NC group, letter next to asterisk indicates tissue (P<0.05).

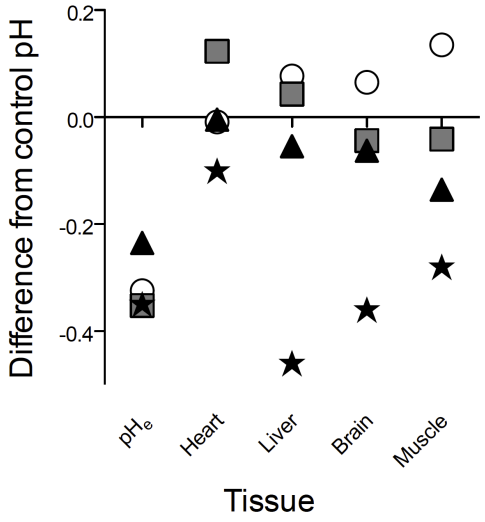

Figure 4.6: Difference in blood and tissue pH of turtles during development following exposure to hypercarbia relative to normocarbia in common snapping turtles (*Chelydra serpentina*) (70 and 90% of incubation and yearlings; this study) and adult western painted turtles (*Chrysemys picta bellii*) (Wasser et al., 1991). Control pH values for pH_e, and pH_i of heart, liver, brain and muscle were subtracted from the values determined following either 1 h HC13 (13 kPa PCO_2, 9 kPa PO_2) exposure (from Figure 4.1) in snapping turtles or 1 h 6.5 kPa PCO_2 exposure in western painted turtles. Mean control (normocarbic) pH was subtracted from individual hypercarbic values to calculate a mean; differences are shown as means only to visualize the large reductions in pH_i in adult turtles compared to either embryos or yearlings in the present study. 70% of incubation (○), 90% of incubation (■), yearlings (▲) and adult western painted turtle (★).

Table 4.1: Plasma ion concentrations at 90% of incubation in *Chelydra serpentina* embryos reared in NC and HC3.5

	Na^+ (mmol l^{-1})	K^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
NC	129.0±3.7	3.5±0.1	116.4±3.4	1.4±0.1
HC3.5	135.0±3.2	3.9±0.1*	117.0±3.7	1.4±0.1

NC, normocarbia (0.03 kPa PCO_2, 21 kPa PO_2; N=8) and HC3.5, hypercarbia (13 kPa PCO_2, 9 kPa PO_2; N=6) reared in (common snapping turtles) embryos.

Data are means ± s.e.m. *Significance between rearing conditions (P<0.05).

Chapter 5: American Alligator Embryos Tightly Regulate Intracellular pH During a Severe Acidosis

5.1 Introduction

Acid-base regulation in adult amniotes relies on net H^+ exchange with the environment through ventilatory and/or renal pathways (Cameron, 1989a); however, during embryonic development of oviparous animals this is constrained as the egg shell structure limits environmental interaction (Eme and Crossley, 2015; Erasmus et al., 1971; Everaert et al., 2011). Acid-base balance is one of the most important physiological parameters and tight pH regulation is critical as small deviations can have large effects on molecular function, and ultimately reduce whole animal performance (Putnam and Roos, 1997). In adult amniotes, compensation of intracellular pH (pH_i) following an acid-base disturbance is usually more rapid than that of blood pH (extracellular pH; pH_e) but compensation in both compartments is coupled, termed coupled pH regulation. Complete pH_i recovery during a sustained respiratory acidosis occurs only following approximately >50% pH_e compensation (Shartau et al., 2016b; Shartau et al., 2016a). Thus, acid-base regulation in adult amniotes is characterized by the regulation of pH_e, which ensures pH_i is protected (Shartau et al., 2016a) – the response in embryonic amniotes constrained within an egg shell is poorly understood (Eme and Crossley, 2015; Everaert et al., 2011). Limited evidence from snapping turtle embryos *Chelydra serpentina* during severe acute respiratory metabolic acidosis suggests that the pattern of acid-base regulation in embryos may differ from adults. Exposure to an acute elevated environmental CO_2 tension (hypercarbia) of *C. serpentina* embryos at two developmental ages resulted in a dramatic reduction in pH_e; however, pH_i was observed to be protected (Shartau et al., 2016b). This trait is referred to as preferential pH_i regulation and has only previously been observed in a few adult anamniotes (e.g. white sturgeon [*Acipenser transmontanus*], armoured catfish [*Pterygoplichthys pardalis*] and greater siren [*Siren lacertina*]), but never in adult amniotes (Shartau and Brauner, 2014; Shartau et al., 2016b).

Vertebrates capable of preferential pH_i regulation exhibit no detectable pH_i reduction during a severe respiratory acidosis, and notably, pH_e compensation is not required for pH_i protection (Shartau et al., 2016a). Regulation of pH_e for coupled pH regulation is limited in embryos due to barriers created by the eggshell and associated membranes (e.g. chorioallantoic membrane) (Erasmus et al., 1971; Everaert et al., 2011; Shartau et al., 2016a). Additionally, the absence or incomplete formation of an extracellular compartment and necessary cardiorespiratory and renal structures, limit pH_e regulation (Eme and Crossley, 2015; Everaert et al., 2011; Shartau et al., 2016b); consequently, preferential pH_i regulation may be a more common trait in reptilian embryos as observed in *C. serpentina*, particularly those that are known to be tolerant of conditions that induce an acid-base disturbance such as elevated CO_2.

The nests of some reptilian species naturally experience large increases in CO_2 and reductions in O_2 (Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984); this may create challenges for acid-base regulation and be further constrained by limited nest and eggshell diffusion (Erasmus et al., 1971). It has been documented that the mound nests of crocodilians can naturally experience CO_2 levels of 2-8.5 kPa (Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984). Chronic high CO_2 tensions may not adversely affect crocodilian embryos during rearing (Eme and Crossley, 2015), but nothing is known about their acid-base status during either chronic or acute CO_2 exposure (Everaert et al., 2011). We were interested in determining whether embryonic crocodilians can protect tissue pH during acute hypercarbia hypoxia, similar to that observed in *C. serpentina*. I hypothesized that *Alligator mississippiensis* embryos would preferentially regulate pH_i during a severe acute respiratory acidosis. This hypothesis was tested by exposing embryos to a severe acute respiratory metabolic acidosis to examine the impact of this exposure on blood and tissue acid-base status to gain insight into the pattern of pH regulation in embryonic crocodilians and determine whether preferential pH_i regulation may be a general trait of CO_2 tolerant reptilian embryos.

5.2 Methods

5.2.1 Subjects of study

Alligator mississippiensis embryos were collected from the Rockefeller Wildlife Refuge at Grand Chenier, LA, USA and transported to the laboratory at the University of North Texas. Embryos were staged to determine approximate age of each clutch (72 d total incubation period at 30^{o}C) and were incubated at 30^{o}C in a walk-in incubator ensuring all embryos developed as female. All embryos were placed in plastic containers and placed in a bed of moist vermiculite mixed in a 1:1 ratio of vermiculite to water. Water content of vermiculite was maintained by weighing the box twice weekly and adding water as needed to keep the mass constant.

5.2.2 Surgical procedures

Embryos were removed from the incubation chamber and candled to identify a tertiary chorioallantoic membrane (CAM) artery. They were then placed in a temperature-controlled surgical chamber (30^{o}C) under normoxic/normocarbic conditions and ~1 cm^{2} of the eggshell was removed under a dissection microscope (Leica MZ6 Leica Microsystems, Waukegan, IL). A tertiary CAM artery was isolated for arterial pressure monitoring and blood sampling. An occlusive catheter was inserted into the vessel using heat-pulled, heparinized, and saline-filled PE-50 tubing, as previously described (Crossley and Altimiras, 2005). The surgical preparations were minimally invasive and no anesthesia/analgesia is required; the entire surgical procedure took 7-10 minutes. Following catheterization, the catheter was fixed to the shell with cyanoacrylic glue and the embryo was placed in a water-jacketed multi-chamber experimental unit (~700 cm^{3} per chamber, one embryo per chamber) and allowed to acclimate for at least 60 minutes. Temperature in the chambers was maintained at 30^{o}C with a circulating water bath (VWR International, LLC, West Chester, PA, USA). Each chamber consisted of a container fitted with a lid with three ports that allowed the catheter and airlines to enter the chamber. To prevent changes in chamber temperature due to incoming air flow, all incoming gas traversed a 1 m copper line submerged within the constant temperature circulator's water bath. Air was forced into each chamber at a flow of 200 ml min^{-1}. Each

arterial catheter was attached to a pressure transducer 1-3 cm above the egg via saline-filled PE50 tubing, connected to an amplifier, and the pressure signal acquired at 40 Hz using PowerLab data recording system (ADInstruments, CO, USA) connected to a computer running ChartPro software (v 7.4 ADInstruments CO, USA). Pressure transducers were calibrated prior to each measurement period with a vertical column of saline, and heart rate was determined with a software tachograph that integrated the arterial pressure trace. Cardiovascular measurements were made to quantify cardiovascular changes during acid-base challenge.

5.2.3 Experimental treatment and physiological measurements

Embryos reared in normocarbic/normoxic (0.03 kPa PCO_2, 21 kPa PO_2; air) were removed at 70% of incubation and subjected to either 1 h exposure to air or hypercarbic hypoxia (13 kPa PCO_2 and 9 kPa PO_2; H13). This treatment was chosen as previously it has been shown to induce a sufficiently severe acidosis (Andrewartha et al., 2014), allowing for the determination of preferential pH_i regulation (Shartau et al., 2016b); the exposure time of 1 h was chosen as pH_e and pH_i typically reach maximal depression at that point (Baker et al., 2009a). The conditions for H13 were generated using compressed O_2, CO_2, and N_2 regulated with mass flow controllers for nitrogen, oxygen and carbon dioxide (GFC Aalborg; Orangeburg, NY, USA) regulated with a command module (Model SDPROC, Aalborg; Orangeburg, NY, USA) to achieve the desired gas mix. O_2 and CO_2 levels were monitored with analyzers (S-A/I and CD-3A, respectively; Ametek Applied Electrochemistry, IL, USA). Gas composition in the chamber changed within 60-120 seconds. Following exposure two aliquots of blood, approximately 150-300 μL, were sampled from the CAM artery by disconnecting the pressure catheter with blood passively flow into a 1 mL heparinized syringe. Blood pH (pH_e; model BMS 3 MK 2; Radiometer) and total CO_2 (TCO_2) (model 965 Analyzer; Corning) were measured immediately using the first aliquot and the second aliquot was centrifuged (3 min at 10,000 rpm), plasma removed and red cells frozen for later analysis of pH_i. Embryos were then euthanized with an overdose of sodium pentobarbital (100 mg kg^{-1}) injected into the CAM artery. Tissues (heart, brain, liver, white muscle and kidney) were then quickly dissected (within 5 min), placed in micro-centrifuge tubes, frozen in liquid

nitrogen and stored at -80°C for later measurements of pH_i. Tissue was later ground under liquid nitrogen and pH_i was measured using the metabolic inhibitor tissue homogenate method (MITH; see Appendix for detailed description of this method); this technique has been validated (Baker et al., 2009b; Portner et al., 1990) and has been previously used in reptiles (Galli and Richards, 2012; Shartau et al., 2016b). Red blood cell (RBC) pH_i was measured using the freeze-thaw technique (Baker et al., 2009a).

5.2.4 Calculations and statistical analyses

Plasma [HCO_3^-] and PCO_2 were calculated using measured TCO_2 and pH values as previously described by Brauner et al. (2004). The CO_2 solubility coefficient and pKa were calculated using equations from Heisler (1984) which were adapted, and experimentally validated, for use with reptile blood (Stabenau and Heming, 1993). Comparison of acid-base changes between control and treatment were conducted using both pH and proton concentration ([H^+]); this was done to mitigate concern regarding the perceived problem of using pH, a logarithmic value, in statistical analyses (Boutilier and Shelton, 1980). [H^+] was calculated from individually measured pH values ($[H^+]=10^{-pH}$) and plotted as mean ± s.e.m.

All data was analyzed using GraphPad Prism v5.0 (GraphPad Software Inc., 2007). Differences between the acid-base parameters of air and H13 exposed groups were compared using a two-sample t-test ($P< 0.05$). Mean arterial pressure (kPa) and mean heart rate (beats min^{-1}) were calculated from the individual mean values for embryos in each exposure group and were based on stable individual mean values for 45 min during the exposure period. Absolute blood pressure was corrected for the pressure transducer's distance above the egg. Mean blood pressure and heart rate were compared using a two-sample t-test ($P< 0.05$). All values are presented as mean ± s.e.m.; sample sizes are N=6-7 except for RBC where N=5.

5.3 Results and discussion

Alligator mississippiensis embryos preferentially regulate pH_i despite a reduction in pH_e during a severe acute respiratory metabolic acidosis. Following 1 h exposure to H13, blood PCO_2 increased from 3.3 ± 0.5 to 8.6 ± 1.0 kPa PCO_2, which was accompanied by a large reduction in pH_e from 7.516 ± 0.027 to 7.010 ± 0.019; blood $[HCO_3^-]$ did not differ (Fig. 5.1A). Despite pH_e being reduced by 0.506 pH units, pH_i of tissues was not reduced; heart and brain pH_i increased (6.346 ± 0.051 to 6.572 ± 0.066 and 6.512 ± 0.046 to 6.693 ± 0.061 pH units, respectively) (Fig. 5.1B), while no change in pH_i of liver, white muscle, or kidney were observed (Fig. 5.1C).

As pH is a measure of $[H^+]$, the changes in $[H^+]$ of blood and tissues following this acidosis reflected those of pH. Blood $[H^+]$ increased from 30.9 ± 1.9 to 80.1 ± 3.3 nM, which was accompanied by reductions in $[H^+]$ of heart and brain (Fig. 5.1D); no change in $[H^+]$ of liver, white muscle or kidney occurred (Fig. 5.1E). Using $[H^+]$ did not yield different statistical conclusions compared to using pH and thus indicate that despite the logarithmic nature of pH, use of pH should not be an issue in these analyses. This is corroborated by Boutilier and Shelton who conclude that the use of pH is as valid of that of $[H^+]$ for statistical analysis; they suggest this conclusion is applicable to all vertebrates that have fairly precise pH (or $[H^+]$) regulation (Boutilier and Shelton, 1980). Our calculations, along with Boutilier and Shelton (1980), provide additional reassurance regarding the acceptability of using pH in statistical analyses in this study, and others.

Exposure to elevated CO_2 was expected to reduce pH_e, however, a pure respiratory acidosis would be associated with an increase in plasma $[HCO_3^-]$ along the blood buffer line from 19.5 mM to 26.4 mM based on a blood buffer value of -16 mM HCO_3^- pH $unit^{-1}$ from chicken embryos (Burggren et al., 2012) (Fig. 5.1A). That $[HCO_3^-]$ is well below the blood buffer line is indicative of net acid excretion from the intracellular to the extracellular compartment and is a characteristic of preferential pH_i regulation (Harter et al., 2014; Shartau et al., 2016a). The RBC were the only tissue to exhibit a reduction of pH_i which is a common trait among fish that preferentially regulate pH_i (Harter et al., 2014). While clearly the RBCs do not actively regulate pH_i in the way that other tissues do, they have a tissue buffer value similar to tissues such as the heart

and brain (Wood and LeMoigne, 1991) and thus inform on the reduction in pH_i that might be expected in tissues lacking the capacity for preferential pH_i regulation (Harter et al., 2014). Differences amongst tissues for pH_i regulation may reflect varying capacity for preferential pH_i regulation; this may be tissue specific or reflect differential organ maturation at 70% of incubation (Shartau et al., 2016b).

The capacity for preferential pH_i regulation may depend on the relative importance of the respective tissue to the physiology of the embryo at this developmental stage. For example, the capacity of heart to regulate its pH_i may underlie the constant blood pressure and heart rate (0.65 ± 0.05 kPa and 69.5 ± 6.3 beats min^{-1}, respectively;) during acute H13 exposure. Preferential pH_i regulation in the heart is also likely responsible for maintaining cardiac function in embryonic *C. serpentina* during acute hypercarbia hypoxia at 70% of incubation but at 90% of incubation cardiac function was reduced (Shartau et al., 2016b). Similarly, preferential pH_i regulation is suggested to preserve cardiac performance in adult white sturgeon (Baker et al., 2011) and armoured catfish (Hanson et al., 2009) during exposure up to 3 and 5 kPa PCO_2, respectively; however, beyond those CO_2 tensions those fishes exhibited modest reduction in cardiac performance. The ability of *A. mississippiensis* embryos to protect (and elevate) pH_i while preserving cardiac function at this developmental stage, suggests they possess a robust capacity to tolerate this respiratory metabolic acidosis, and that the impact on whole embryo performance, at least acutely, is minimal.

In white sturgeon, PCO_2 tensions greater than 6 kPa PCO_2 are also associated with a reduction in metabolic rate while still preferentially regulating pH_i (Baker and Brauner, 2012). Other studies have shown metabolic depression in response to hypercarbia-induced respiratory acidosis (Baker and Brauner, 2012; Michaelidis et al., 2005; Stapp et al., 2015) and that the response, including protein synthesis, differs between tissues and organisms (Stapp et al., 2015). During hypercarbia in the peanut worm (*Sipunculus nudus*), there is a metabolic depression which is associated with a shift to less ATP costly ion-transporters which allow for compensation of the accompanying intracellular acidosis (Portner et al., 1998; Portner et al., 2000). Given the differential response of pH_i in *A. mississippiensis* embryos in response to hypercarbia hypoxia, it may be that tissues are affected differently by the extracellular acidosis. The protection of pH_i

may be reflected in the relative ability to shift to the optimal acid-base ion transporters, such that the most acid sensitive tissues such as brain and heart, possess the greatest capacity for this and thus exhibit the most robust capacity for pH_i regulation against reduction in pH_e.

Embryonic *A. mississippiensis*, like embryonic turtles in Chapter 4, were subjected to both respiratory and metabolic acidosis as a consequence of hypercarbia and hypoxia, respectively. As hypoxia leads to anaerobic metabolism and the increased production of lactate, this may have affected tissues differently. While lactate concentration was not measured, exposure of similar stage embryonic chickens to 1 h hypercarbia hypoxia resulted in a pH_e reduction of ~0.8 pH unit and increase in blood lactate from 0.8 to 14 mM (Andrewartha et al., 2014), intracellular pH or lactate were not measured so it is not known how the tissues responded but they likely experienced large pH_i reduction and lactate increase. The response of pH_i to hypercarbia or hypoxia alone in amniote embryos are unknown; consequently, the possible differences between them on pH_e and pH_i should be considered.

Preferential pH_i regulation likely confers the CO_2 tolerance exhibited by *A. mississippiensis* embryos, which may enhance embryonic survival by allowing them to tolerate acid-base disturbances in an environment that is not favorable to net acid excretion. The ability of *C. serpentina* and *A. mississippiensis* embryos to preferentially regulate pH_i, species where adults use coupled pH regulation (Shartau et al., 2016a; Wasser et al., 1991), suggests the pattern of acid-base regulation is modified during reptilian development. Compared to older embryos and post-hatch animals, the capacity for pH_i regulation in turtles appears to be the greatest at 70% of incubation (Fig. 5.2). The differential ability of embryos and post-hatch reptiles to regulate pH_e and pH_i (only pH_i for brain and muscle are shown) following an acute acidosis is shown in Figure 2. Although pH_e is consistently reduced, pH_i is well regulated in 70% of incubation embryos, but in post-hatch turtles (*C. serpentina* and *C. picta bellii*) and lizards (*A. equestris* and *D. dorsalis*), pH_i is reduced.

These results show that embryonic *A. mississippiensis* fully protect pH_i against severe reduction in pH_e during hypercarbia hypoxia, corroborating results in turtle (Shartau et al., 2016b), further demonstrating that preferential pH_i regulation occurs in

reptilian embryos; a strategy of acid-base regulation that has only been identified in a few adult anamniotes vertebrates (Shartau et al., 2016a). Although more work is needed to support the hypothesis recently proposed by Shartau et al. (2016a) that preferential pH_i regulation is an embryonic strategy of acid-base regulation in vertebrates, these findings in *A. mississippiensis* provide the second example of this strategy in an embryonic reptile, and the first in a crocodilian. Further studies are required to assess whether *A. mississippiensis* embryos exhibit a similar transition from preferential pH_i regulation to coupled pH regulation like that in turtles, and whether this strategy of acid-base regulation manifests in non-reptilian embryonic vertebrates.

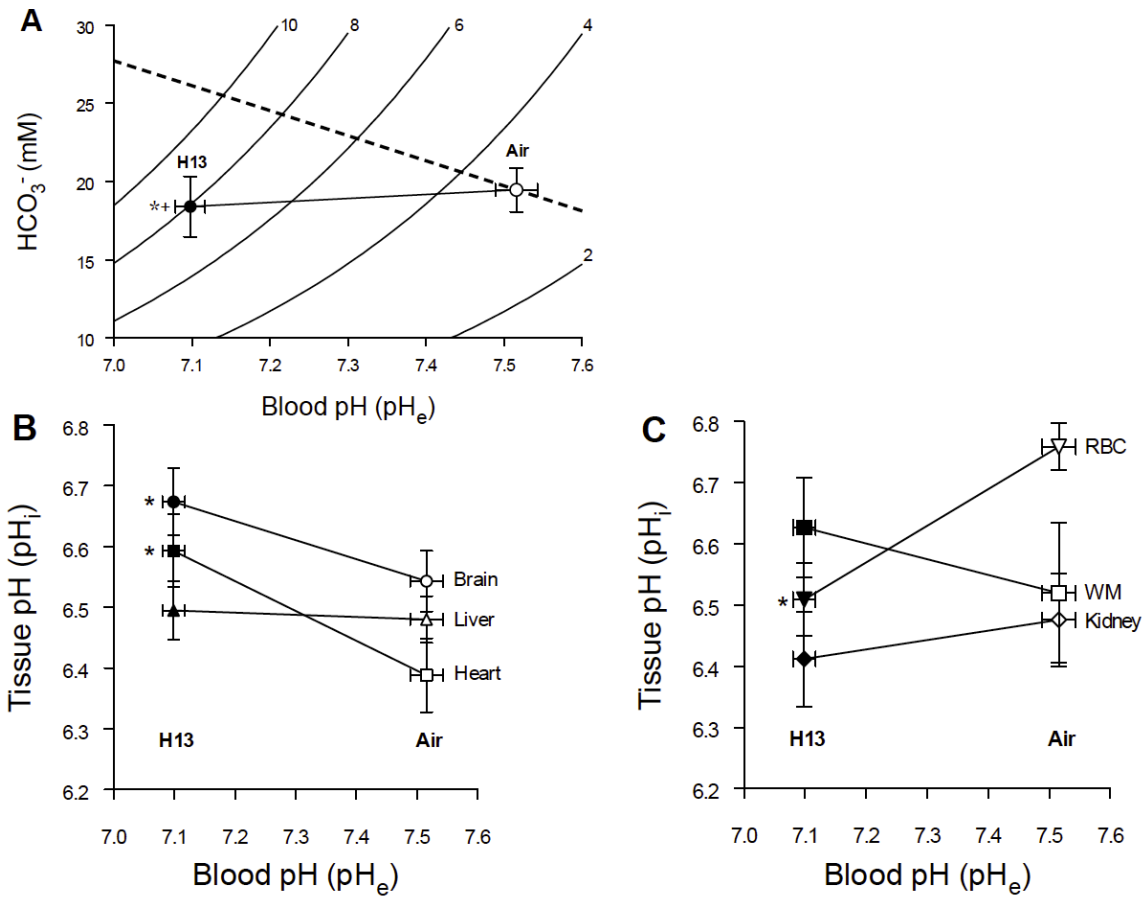

Figure 5.1: Effect of exposure to an acute respiratory acidosis in *Alligator mississippiensis* embryos on blood and tissue acid-base status. Blood pH (pH_e), blood [HCO_3^-] are presented on a pH-HCO_3^- plot. Embryos at 70% of incubation were sampled in air or following a 1 h hypercarbic hypoxia (13 kPa PCO_2, 9 kPa PO_2: H13) exposure; curved lines represent PCO_2 isopleths and dashed line represents the non-bicarbonate blood buffer value (adapted from Burggren et al., 2012) (A). Relationship between blood pH (extracellular pH [pH_e]) and tissue pH (pH_i) (B, C) or relationship between in air or blood proton concentration [H^+] (nM) and tissue [H^+] (D, E) following 1 h exposure to H13. Brain (●), liver (▲) and heart (■) (B, D), and red blood cells (RBC, ▼), white muscle (WM, ■) and kidney (◆) (C, E). Values are presented as means ± s.e.m. Symbols (*, +) indicate significant differences ($P<0.05$) between air (open symbols) and H13 (closed symbols) treatment for pH or [H^+] (*) and blood HCO_3^- (+).

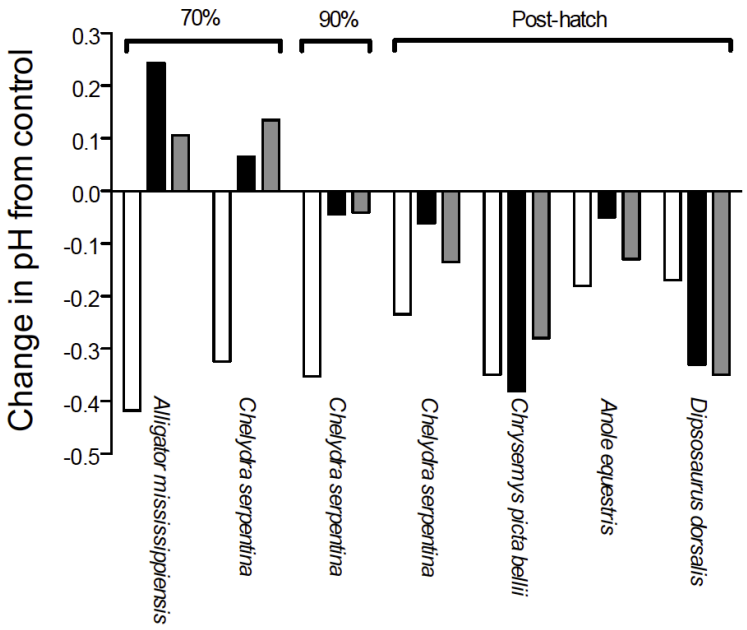

Figure 5.2: Difference in blood pH (pH_e) and tissue pH (pH_i) during development following a respiratory acidosis in embryonic *Alligator mississippiensis* (American alligator; 70% to hatch) and *Chelydra serpentina* (snapping turtle; 70 and 90% to hatch), and in post-hatch *C. serpentina*, *Chrysemys picta bellii* (western painted turtle), *Anolis equestris* (knight anole) and *Dipsosaurus dorsalis* (desert iguana). Control pH values for pH_e (open bar), and pH_i of brain (black bar) and muscle (grey bar) were subtracted from the values determined following either 1 h exposure to 13 kPa PCO_2, 9 kPa PO_2 in *A. mississippiensis* (this study) and *C. serpentina* (Chapter 4), or 1 h dive which increased arterial PCO_2 to 6.5 kPa in *C. picta bellii* (Wasser et al., 1991), or 1 h exposure to 5 kPa PCO_2 in *A. equestris* and *D. dorsalis* (Snyder et al., 1995). Differences are shown as means only to visualize the changes across developmental ages. Values that are ≥0 are representative of preferential pH_i regulation while those that are <0 indicate a net acidosis.

Chapter 6: General Discussion and Conclusions

The objective of my thesis was to investigate the usage, distribution/prevalence, and origin of preferential pH_i regulation as a strategy of acid-base regulation in vertebrates. This objective was addressed by investigating how adult fishes and embryonic amniotes respond to severe acute acid-base disturbances. Chapter 2 suggests that preferential pH_i regulation is not used by all tissues to protect against all types of acidosis in white sturgeon. Chapter 3 indicates that preferential pH_i regulation occurs in many fish species and in numerous fish phylogenetic groups in response to severe acute hypercarbia. Finally, Chapters 4 and 5 demonstrate that preferential pH_i regulation occurs in reptile embryos and may be lost in adults. Together, these results demonstrate that preferential pH_i regulation is a widely used strategy of acid-base regulation amongst vertebrates in response to severe acute hypercarbia. Additionally, it may be an embryonic strategy that is either retained or lost in adults, and that differences exist in the degree of pH_i protection between the tissues in response to an acid-base disturbance, and differences in pH_i regulation occur between different types of acute acid-base disturbances.

This General Discussion will examine preferential pH_i regulation as a distinct pattern of acid-base regulation compared to the more familiar/traditional strategy of coupled pH_e/pH_i regulation. The significance of using preferential pH_i regulation will be explored, including how it may have played a critical function in a number of key transitions in vertebrate evolution.

6.1 Thesis overview and major contributions

Chapter 2 investigated whether preferential pH_i regulation is a general strategy of acid-base regulation in response to different types of acidoses. The goal of this chapter was to determine if preferential pH_i regulation could be assessed in fishes using various acid-base challenges such as exhaustive exercise, in addition to hypercarbia where it has

been observed previously (Baker et al., 2009a). If different acidoses can be used to demonstrate the presence or absence of preferential pH_i regulation, this would allow for the use of acid-base exposures other than hypercarbia (e.g. exhaustive exercise) to conduct the survey of presence or absence of preferential pH_i regulation in Chapter 3. Additionally, inference regarding the presence or absence of preferential pH_i regulation from the literature could occur as most acid-base challenges are not conducted using hypercarbia. I hypothesized preferential pH_i regulation is a general acid-base regulatory strategy in *Acipenser transmontanus* (white sturgeon). The results from Chapter 2 indicated that *A. transmontanus*, which preferentially regulate pH_i during hypercarbia, do not exhibit the same capacity for pH_i regulation in all tissues during metabolic acidoses induced by anoxia, exhaustive exercise and air exposure. This suggests that response between acidoses varies and thus, metabolic acidoses may not indicate whether fishes possess the capacity for preferential pH_i regulation. These results help shape subsequent chapters by emphasizing the importance of consistently using the same type of acid-base disturbance (i.e. hypercarbia) to assess the presence or absence of preferential pH_i regulation.

Chapter 3 investigated the presence or absence of preferential pH_i regulation in fishes. The objective of this chapter was to determine if preferential pH_i regulation is a common and widespread strategy of acid-base regulation amongst fishes as prior to this thesis preferential pH_i regulation had only been observed in three fishes (*A. transmontanus, Pterygoplichthys pardalis, Synbranchus marmoratus*). I hypothesized that preferential pH_i regulation would occur in fishes tolerant of severe acute hypercarbia; this was supported by the results. Chapter 3 shows that an additional 15 fish species use preferential pH_i regulation; these fishes include water and air breathers, as well as tropical and temperate species (Table 6.1), and span numerous phylogenetic groups (Fig. 6.1). These findings represent a major contribution to understanding the distribution of acid-base regulatory strategies in fishes as preferential pH_i regulation can now be considered relatively common (or at least, not rare) and broadly used.

As preferential pH_i regulation was demonstrated in Chapter 3 to be closely associated with tolerance to severe hypercarbia in adult fishes, I was interested in investigating whether this strategy might be used by amniotes, some of which are highly

hypercarbia tolerant during embryonic development. Chapter 4 investigated the pattern of acid-base regulation during development of a hypercarbia tolerant amniote, *Chelydra serpentina* (common snapping turtle). I hypothesized that embryonic turtles would preferentially regulate pH_i. Results from this chapter indicate that embryonic turtles preferentially regulate pH_i and that capacity for pH_i regulation is reduced throughout development (Fig. 6.2). These findings are highly significant as they demonstrate for the first time that preferential pH_i regulation occurs in an amniote, and that the pattern of acid-base regulation changes throughout ontogeny. This suggests that preferential pH_i regulation may be an embryonic pattern of acid-base regulation and that preferential pH_i regulation may be retained or lost in adults.

Chapter 5 investigated acid-base regulation in another hypercarbia tolerant amniote, *Alligator mississippiensis* (American alligator), to see if I could corroborate findings from Chapter 4; i.e. whether other amniote embryos use preferential pH_i regulation. As alligator embryos are hypercarbia tolerant, I hypothesized that embryos will preferentially regulate pH_i; this is supported by the results. The importance of this finding is that it demonstrates preferential pH_i regulation occurs during development in another, distantly related, amniote species, which further strengthens support for the hypothesis that preferential pH_i regulation is an embryonic pattern of acid-base regulation. Together, these chapters greatly expand the understanding of preferential pH_i regulation as strategy of acid-base regulation that provides exceptional pH_i protection during hypercarbia-induced respiratory acidoses in a large number of diverse fishes, and may represent an embryonic strategy as indicated by its use in embryonic amniotes.

6.2 Preferential pH_i regulation: A common and distinct pattern of acid-base regulation

Before this dissertation, preferential pH_i regulation was considered to be a novel and rare pattern of acid-base regulation (Fig. 1.4) (Fig. 1.4; Brauner and Baker, 2009; Brauner et al., 2004); however, based upon my findings in this thesis, this does not appear to be the case. The findings in Chapter 4 and 5 that preferential pH_i regulation is

used by developing amniotes was intriguing but perhaps is expected given the putative limitations embryos face during development. While the mechanisms remain yet to be uncovered, use of preferential pH_i regulation allows embryos to compensate for acid–base challenges to pH_i, despite the incomplete formation of the extracellular compartment and associated structures (e.g. cardiovascular, respiratory, and renal systems) that are required for coupled pH regulation.

Embryos may also experience additional challenges due to encapsulation within extra-embryonic structures (e.g. eggshells or egg capsules) (Goldberg et al., 2008; Tazawa, 1980). These structures typically permit the perfusion of O_2 and CO_2 but create diffusion gradients (Ciuhandu et al., 2007; Goldberg et al., 2008; Tazawa, 1980); additionally, in the aquatic, but not terrestrial environment, limited exchange of ions may occur (Alderdice, 1988; Everaert et al., 2011) depending on the permeability of the specific extra-embryonic structure. Reduced exchange of gases and acid-base ion equivalents with the external environment may putatively limit the ability of encapsulated embryos to use coupled pH regulation (Erasmus et al., 1971). The results in Chapters 4 and 5, along with the physical limitations posed by encapsulation, led me to hypothesize that preferential pH_i regulation may represent the basal pattern of acid–base regulation in vertebrates as an acid–base regulatory strategy during development, with adults either retaining or losing this trait. Thus, preferential pH_i regulation may in fact be ubiquitous amongst vertebrates (and possibly invertebrates, which can face many of the same challenges as vertebrates) (e.g. Kikkawa et al., 2008; Portner et al., 1998; Spicer et al., 2007; Spicer et al., 2011). Further studies should examine other embryos at various developmental stages to 1) assess how they respond to acid-base challenges, 2) determine at which developmental stage preferential pH_i regulation is lost, and 3) assess the role of encapsulation on the pattern of acid-base regulation (e.g. compare encapsulated and free-swimming embryos).

If preferential pH_i regulation is an embryonic trait, then the occurrence of preferential pH_i regulation in adult species may represent the retention of the embryonic trait; whereas, species exhibiting only coupled pH regulation would imply the loss of preferential pH_i regulation and the acquisition of coupled pH regulation, which may represent a derived strategy of acid-base regulation. In comparison to the start of this

thesis (Fig. 1.4), the number of species identified to use preferential pH_i regulation has increased greatly, which represents a fundamental shift in the quantity and phylogenetic distribution of species using preferential pH_i regulation. Based upon my work (in collaboration with others) preferential pH_i regulation has been identified for the first time in adult lamprey (*Entosphenus tridentatus*), elasmobranchs (e.g. *Potamotrygon* spp.), basal sarcopterygians (e.g. *Lepidosiren paradoxa*), and numerous additional species have been included in groups where only a single species had previously been known to use preferential pH_i regulation (e.g. basal actinopterygians, Siluriformes, Perciformes). The diversity of these species includes tropical, subtropical and temperate species; additionally, there is a mix of water and bimodal breathers (Table 6.1). This suggests preferential pH_i regulation is not restricted to a particular taxonomic group, geographic area or mode of respiration, and it may be that the physical characteristics of the environment are strong selectors for this pattern of acid-base regulation.

As preferential pH_i regulation was believed to be a rare strategy at the start of this thesis, I specifically targeted fishes that would likely live in high CO_2 environments as they would be more likely to use preferential pH_i regulation; this approach likely created a bias towards identifying species that preferentially regulate pH_i compared to those using coupled pH regulation in Chapter 3. All the fishes sampled here primarily reside in freshwater, which is likely the more challenging environment for acid-base regulation compared to marine environments, due to the greater likelihood of severe hypercarbia (Brauner and Baker, 2009; Furch and Junk, 1997; McNeil and Sasse, 2016; Raymond et al., 2013). If marine fishes were sampled, it is likely this would include many coupled pH regulators as the marine environment is typically more stable and the availability of acid-base relevant counter ions for pH_e regulation would allow for the loss of preferential pH_i regulation as coupled pH regulation would be feasible. However, some marine environments may pose challenges for acid-base regulation, such as the intertidal zone (Richards, 2011) and near deep-sea CO_2 vents (Ishimatsu et al., 2008); these could be environments that select for the retention of preferential pH_i regulation in adults. Despite that my selection of species likely underrepresented the prevalence of coupled pH regulation, that preferential pH_i regulation was identified in so many species was

unexpected and represent an important finding demonstrating that it is relatively common and widespread among fishes.

The putative retention of preferential pH_i regulation in many adult vertebrates is likely influenced by the environment. Many adult fishes using preferential pH_i regulation inhabit environments characterized by challenging conditions for acid-base regulation. For example, Amazonian fishes (e.g. *Colossoma macropomum*) live in ion-poor waters with naturally low pH that experience large fluctuations in water PCO_2 and PO_2 (Pinardi et al., 2014; Val et al., 2005). Using preferential pH_i regulation may be the only viable solution to the acid-base challenges associated with these environments; particularly under conditions such as severe acute hypercarbia, it is highly unlikely that compensation of pH_e could occur. Conversely, the putative loss of preferential pH_i regulation in some adult vertebrates may reflect the fact that they inhabit environments where pH_e can be sufficiently regulated. For example, fishes such as Atlantic cod and rainbow trout typically reside in normocarbic, normoxic waters with sufficient ions for acid-base relevant ion exchange that allow them to easily compensate pH_e during the acid-base challenges they may experience. Similarly, adult amniotes (e.g. turtles and alligators), which are terrestrial air breathers, are able to adjust air convection requirements to compensate for an acid-base disturbance. While the direct involvement of the environment in influencing the pattern of acid-base regulation has not been thoroughly assessed, some work on this has been conducted. Environmental influences on acid-base regulation have been implicated in *O. mykiss* exposed to hypercarbia in waters containing different ionic composition which resulted in large differences in the degree and speed of pH_e compensation (Larsen and Jensen, 1997). In *Pangasianodon hypophthalmus* exposed to hypercarbia, they were observed to preferentially regulate pH_i but also compensate pH_e in unaltered pond water; however, when placed in hypercarbia in pond water with artificially lowered pH, they only regulated pH_i and not pH_e (R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong, M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong, and C.J.B., unpublished observations and reviewed in Shartau et al., 2016a); this is consistent with the idea of environmental influence on the pattern of acid-base regulation. This indicates that patterns of pH regulation are not fixed, and that animals can switch between coupled pH regulation and preferential pH_i regulation, and that pH_e regulation is

vulnerable to changes in environmental parameters. Whether patterns of pH regulation are fixed/determined or if animals can switch between coupled pH regulation and preferential pH_i regulation is unknown, but it does appear that pH_e regulation itself is vulnerable to environmental changes.

6.2.1 Inter- and intra-specific variation of preferential pH_i regulation

Amongst preferential pH_i regulators, the capacity for pH_i regulation is not uniform. While Chapter 2 demonstrated that *A. transmontanus* do not protect all tissues against metabolic acidoses (Shartau et al., 2017a), a similar study on *P. pardalis* indicated that preferential pH_i regulation is a general strategy as they protected pH_i following exhaustive exercise, anoxia and metabolic alkalosis (Harter et al., 2014). As both *A. transmontanus* and *P. pardalis* exhibit tremendous pH_i protection during hypercarbia it is unclear as to why the former has less control over pH_i during metabolic acidoses; however, it may be associated with differences in their capacity for pH_e regulation. Unlike *A. transmontanus*, *P. pardalis* appear to have little to no capacity for pH_e regulation, as evident from the near complete lack of pH_e compensation at all PCO_2 exposures over 24-96 h, including at *ca*. 1 kPa PCO_2 (Brauner et al., 2004); *A. transmontanus* can fully compensate pH_e while preferentially regulating pH_i at 1.5 kPa PCO_2 (Baker et al., 2009a). Thus, tissues in *P. pardalis* may possess a more robust capacity for pH_i regulation to deal with the lack of any pH_e compensation, which could be further underlined by the need to respond to naturally occurring severe acid-base challenges.

The capacity for pH_i regulation varies between tissues, and different species exhibit different degrees of variability in those tissues. In some species that use preferential pH_i regulation, the degree of pH_i regulation varies between tissues, for example, *L. oculatus* exposed to 6 kPa PCO_2 exhibit a significant increase in heart pH_i but no pH change in other tissues (Fig. 3.2B); however, *Atractosteus spatula* exposed to 6 kPa PCO_2 exhibit a significant increase in liver pH_i but no pH change in other tissues (Fig. 3.2C). While most tissues amongst pH_i preferentially regulating species remain unchanged during hypercarbia, Figure 3.2 demonstrates the variation amongst species and tissues. The reason for this variation is not known but it could indicate that those

tissues which are becoming alkalotic relative to normocarbia possess a greater capacity for pH_i regulation and/or that those tissues err on the side of pH_i overcompensation. Conversely, that other tissues do not experience pH_i change during hypercarbia could indicate that they have a greater capacity to tightly maintain intracellular pH homeostasis, which could be the optimal strategy to avoid intracellular disruption. Another possibility is that differences in pH_i regulation amongst tissues reflect animals prioritizing some tissues over others, which may occur if pH_i regulation becomes more challenging as blood acidity increases. In this situation, less critical tissues (e.g. muscle) may be less tightly regulated to avoid exacerbating the pH_e reduction to ensure pH_i of critical tissues (e.g. brain) can be well protected.

These differences in pH_i regulation may reflect differences in cellular mechanisms between tissues. As pH_i regulation is likely nearly instantaneous, based on the *A. transmontanus* heart pH_i response to hypercarbia (Baker, 2010; Shartau et al., 2016a), it is likely that the cellular transporters involved in acid-base regulation are present in the plasma membrane. Transporters could increase in activity as pH_i deviates from the transporter's pH set point, thus triggering pH_i regulation. Indeed, in mammalian cells, increased activation of acid-base transporters such as NHEs and MCTs occurs when pH moves away from the pH set point of steady state pH levels and restoration of pH_i is initiated (Demaurex, 2002; McBrian et al., 2013; Schapiro and Grinstein, 2000; Tokudome et al., 1990). The cellular and molecular mechanism of preferential pH_i regulation remain unknown and thus this remains an important area of future research on this topic.

6.3 Preferential pH_i regulation: A potential developmental and evolutionary strategy to cope with acute acid–base disturbances

Preferential pH_i regulation may be the embryonic strategy of acid-base regulation as it allows embryos to protect their cells and tissues without relying on pH regulation of the external medium (i.e. pH_e); this may be particularly important during severe acid-base disturbances. Retention or loss of preferential pH_i regulation in adulthood may due to

environmental and physiological factors as use of preferential pH_i regulation in more challenging environments is likely to enhance survival; however, many vertebrates appear to lose the capacity for preferential pH_i regulation and acquire coupled pH regulation. The reason for the loss of preferential pH_i regulation is not understood. Preferential pH_i regulation offers exceptional tolerance to severe acute respiratory acid-base disturbances and does not appear to be metabolically costly at the whole animal level during severe hypercarbia exposure (Baker and Brauner, 2012); however, the putative advantages of preferential pH_i regulation may not apply uniformly to metabolic acidoses (Shartau et al., 2017a) and it is not known how well preferential pH_i regulation functions during chronic acid-base disturbances. Furthermore, although the metabolic cost of preferential pH_i regulation has been determined to be low, this was only investigated *A. transmontanus* (Baker and Brauner, 2012); it is not known if this applies to other species. Additionally, there may be costs not yet determined or quantified with having a greater imbalance in pH between the extracellular and intracellular compartments; these could be related to disruption of membrane proteins affecting cellular function.

6.3.1 Preferential pH_i regulation: An exaptation for vertebrate evolution?

Vertebrates are believed to have had a marine origin (Carrete Vega and Wiens, 2012; Halstead, 1985); the marine environment is generally characterized by being relatively stable with respect to PCO_2, PO_2, and temperature (compared to freshwater), which may limit the occurrence of severe environmental acid-base disturbances. Additionally, marine environments are ion-rich, which may have facilitated the use of coupled pH regulation during acid-base challenges; use of this strategy is observed in a basal marine vertebrate, the *Eptatretus stoutii* (Pacific hagfish), an osmo- and iono-conformer, which compensates pH_e and pH_i during a hypercarbic-induced respiratory acidosis (Baker et al., 2015), as well as marine elasmobranches and teleosts (Brauner and Baker, 2009; Shartau et al., 2016a; Wood et al., 1990). The transition of vertebrates from marine to ion-poor fresh water likely posed a challenge for acid-base regulation due to the reduced availability of counter-ions for coupled pH regulation. Consequently, the transition to freshwater may have led to the broader retention of preferential pH_i

regulation in adults. This transition likely occurred approximately 420-430 million years ago in the late Silurian (Halstead, 1985) by the ancestors of the basal euteleostom fishes where global average temperature and atmospheric CO_2 tensions were higher than present day levels (Clack, 2007). These conditions may have resembled present day tropical systems, such as the Amazon River, which have warm, ion-poor, CO_2-rich waters (Furch and Junk, 1997) and may have promoted the retention of preferential pH_i regulation to maintain acid-base homeostasis; thus, protecting against acid-base disturbances that would otherwise be intolerable if relying on coupled pH regulation (Brauner and Baker, 2009; Shartau and Brauner, 2014).

As fishes colonized tropical freshwater environments, preferential pH_i regulation may have been beneficial when encountering severe hypercarbic conditions present in many of these habitats (Furch and Junk, 1997; Heisler, 1984; Li et al., 2013; Ultsch, 1987). Preferential pH_i regulation confers exceptional CO_2 tolerance in nearly all species investigated in this thesis (Chapter 3) and CO_2 tolerance in these environments may be important as hypercarbia is often associated with aquatic hypoxia (Ultsch, 1987); the latter of which is believed to be the primary driver of the evolution of air breathing in fishes (Graham, 1997; Randall et al., 1981). As fishes developed the capacity for air breathing, and became bimodal breathers, the retention of preferential pH_i regulation may provide a means to cope with acid–base challenges associated with air breathing (Shartau and Brauner, 2014). Bimodal breathing fishes take up O_2 from water or air, but typically excrete the majority of CO_2 to the water as the capacitance of water for CO_2 is much greater than air (Graham, 1997; Randall et al., 1981). Consequently, in bimodal breathing fishes, air breathing leads to a rapid increase in blood PCO_2, as CO_2 excretion rates at the gills are reduced due to emersion or reduced gill blood flow and/or gill ventilation. Depending on the species and conditions, blood PCO_2 can increase to >3 kPa PCO_2 during an air-breathing episode causing a reduction in pH_e (Shartau and Brauner, 2014). Air-breathing in fishes is thought to have evolved in tropical environments that likely experience both hypoxia and hypercapnia (Ultsch, 1987); thus, these fishes may have already been subjected to selection pressures to retain preferential pH_i regulation, which could then serve as an exaptation for dealing with an air breathing induced respiratory acidosis (Brauner and Baker, 2009; Shartau and Brauner, 2014).

The transition of vertebrates from water to land posed a number of physiological challenges due to the physical differences between the aquatic and terrestrial environments; one of these is acid-base regulation. Transitioning from an aquatic water breather to a terrestrial air breather involved changes in blood acid-base status as the former have low PCO_2, low plasma [HCO_3^-], and high pH_e, while the latter have high PCO_2, high plasma [HCO_3^-], and low pH_e (Randall et al., 1981; Ultsch, 1996). Additionally, acid-base regulation between the two differs in that water breathers rely on physicochemical buffering and net transport of acid-base equivalents, while air breathers depend mainly on changes in ventilation rate to alter PCO_2 and thus pH (Brauner and Baker, 2009). Exactly how early vertebrates made this transition is not known but it is hypothesized that use of preferential pH_i regulation may have played an important role in minimizing the effects of respiratory acidosis (Brauner and Baker, 2009; Shartau and Brauner, 2014; Shartau et al., 2016a).

Although early terrestrial vertebrates were semi-aquatic bimodal breathers, they still excreted the majority of CO_2 into the water (Janis et al., 2012); therefore, preferential pH_i regulation may have been important to deal with the respiratory acidosis associated with terrestrial excursion when venturing onto land, to forge, escape predation, or related to reproduction. As vertebrates became more dependent on air breathing (i.e. moved from being facultative to obligate air breathers), and terrestrial excursions became longer, the rise in blood PCO_2 would have become greater, resulting in increasingly severe respiratory acidosis (Janis et al., 2012).

The increase in plasma [HCO_3^-] in air breathers may be due to increased renal HCO_3^- production and retention (Gonzalez et al., 2010). Additionally, it has been postulated that highly vascularized dermal bone was involved in providing HCO_3^- to buffer respiratory acidosis (Janis et al., 2012), which may have been used during the terrestrialization of tetrapods as bone and shell contribute to buffering acidosis associated with anoxia in reptiles (Jackson, 2003; Jackson et al., 2000a; Jackson et al., 2000b) and amphibians (Warren, 2005), but not in fish (Harter et al., 2014). Preferential pH_i regulation may have acted as an important intermediate step protecting tissues during these transitory stages. Increased blood PCO_2 would have made excretion of CO_2 into the air easier due to the large diffusion gradient; consequently, tight regulation of pH_e would

be possible via control of ventilatory rate. Unlike water breathers where ventilatory rate is typically O_2 dependent as O_2 is often more limited in aquatic environments, terrestrial air breathers are typically not O_2 limited; thus, control of ventilation is moderated by regulation of blood pH, which is adjusted by adjusting blood PCO_2 by changing air convection requirement (Cameron, 1989a). This, along with the use of the kidneys as the primary organ for acid-base regulation permits tetrapods to tightly control pH_e, and thus maintain pH_i homeostasis. These ideas are highly speculative but presently little is known about this transition in acid-base status.

6.4 Future research directions

This thesis has made a significant advancement to the area of preferential pH_i regulation; yet more work is needed to fully understand this pattern of acid-base regulation, and as such, many interesting areas remain to be examined. Some of the topics for future research I believe are worth investigating are described below.

6.4.1 Survey of fish species

With an estimated 32,000+ fish species (Nelson, 2006), investigating the strategy of acid-base regulation in even a fraction of these species would be a challenging task. However, it would be highly informative if a few additional species were examined, allowing for a better understanding of the diversity (or lack thereof) of acid-base regulatory strategies amongst fishes. While Chapter 3 attempted to include a diverse sample of species, there is still considerable room for improvement in order to gain a broader appreciation for how fishes regulate pH and to avoid overgeneralizing. As previously indicated, many of the species were selected to obtain a high success rate of identifying species using preferential pH_i regulation when I thought it was a relatively rare phenomenon. To obtain a more comprehensive understanding of the strategy of acid-base regulation amongst various groups it would be beneficial to include a few additional species and groups. This thesis has not investigated any marine fishes, which include a phylogenetically diverse range of fishes. Similar to freshwater fishes, it would be

informative to investigate basal and derived marine species, as well as air breathers. A couple of basal marine groups that should be examined in more detail include the Chondrichthyes and coelacanths. Few studies have investigated acid-base regulation in Chondrichthyes in response to severe pH disturbances and almost none have measured both pH_e and pH_i. The coelacanths are the only extant marine sarcopterygian fishes and thus would be fascinating based on their habitat and phylogenetic position (however, obtaining these fishes would be undoubtedly challenging and/or cost prohibitive). There are numerous marine air breathing fishes amongst the teleosts, ranging from the basal to derived including the Elopiformes (*Megalops atlanticus* Atlantic tarpon and *M. cyprinoides* ox eye herring), three families within the Salmoniformes (Umbridae, Lepidogalaxiidae, and Galaxiidae), sculpins in the order Scorpaeniformes (family Cottidae), and mudskippers in the order Perciformes (family Gobiidae) (Graham, 1997). Many of the species investigated in Chapter 3 are tropical or sub-tropical air breathers; one unique air breather that would be interesting to include in this survey would be the Arctic air breathing fish *Dallia pectoralis* (Alaska blackfish) (Lefevre et al., 2014). While the polar environment differs greatly from those in the tropics/sub-tropics, they are subjected to periods of hypoxia as lakes freeze and thus, may be exposed to acid-base challenges where preferential pH_i regulation would be useful. These groups and species are not exhaustive in terms of which fishes might be worth investigating, but hopefully provide some idea where to continue this survey of acid-base regulation in fishes.

6.4.2 Acid-base regulation during development

Similar to the survey in Chapter 3, the discovery of preferential pH_i regulation in embryonic amniotes in Chapters 4 and 5 warrant further investigations into other species. The work in Chapters 4 and 5 has resulted in a new hypothesis that preferential pH_i regulation is an embryonic pattern of acid-base regulation that is either retained or lost in adults; however, additional work is needed to fully support this hypothesis and investigating acid-base regulation in other embryonic vertebrates are needed, including fishes, especially those using coupled pH regulation (e.g. *Oncorhynchus mykiss*). Among other vertebrates, embryonic chickens would be useful to examine as they are well studied with respect to changes in pH_e during hypercarbia but nothing is known about

how pH_i is affected (Andrewartha et al., 2014; Burggren et al., 2012; Everaert et al., 2011; Mueller et al., 2014). Investigating a broad phylogenetic representation of amphibians, reptiles, birds, and mammals during development would be invaluable towards supporting (or refuting) this new hypothesis.

6.4.3 Chronic acid-base disturbances

This thesis has exclusively focused on acute acid-base challenges, yet chronic acid-base disturbances are common and it is largely unknown if preferential pH_i regulation also protects against chronic disturbances. One study examined *Anguilla anguilla* (European eel) during a six-week exposure to hypercarbia at 2, 4 or 6 kPa PCO_2 and found that despite pH_e being only partially compensated at 6 weeks, pH_i of heart and white muscle were protected (McKenzie et al., 2003). This suggests that *A. anguilla* preferentially regulate pH_i during chronic acid-base disturbances (the response during acute hypercarbia is not known in *A. anguilla*). As responses to acute and chronic acid-base disturbances can vary considerably in species using coupled pH regulation, as demonstrated by *O. mykiss* (Brauner and Baker, 2009; Smart et al., 1979). It would be valuable to understand if there are differences in capacity/ability for preferential pH_i regulation between acute and chronic exposures by examining the acute (<48 h) and chronic (>4 weeks) acid-base disturbances.

6.4.4 Role of the environment

As indicated previously, it is hypothesized that the loss or retention of preferential pH_i regulation is determined by environmental and physiological factors. Results from *P. hypophthalmus* suggest that indeed, the environment may be associated with the ability of fishes to regulate pH_e and thus require them to use preferential pH_i regulation (R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong, M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong, and C.J.B., unpublished observations). It has been shown that rainbow trout pH_e regulation is dependent on environmental ion availability (Larsen and Jensen, 1997). Studies investigating the role of the environment on the pattern of acid-base regulation would be informative about the selective pressures for the putative retention of preferential pH_i regulation. Possible experiments could include examining pH_e and pH_i

changes in response to hypercarbia exposure in water with various ion composition, similar to Larsen and Jensen's study (Larsen and Jensen, 1997), with ionic concentrations ranging from ion-poor, such as found in the Rio Negro (Brauner et al., 2004), to that of typical hard water (Lecuyer, 2014). This could also include varying water pH as low water pH inhibits pH_e regulation (Lin and Randall, 1995; Shartau et al., 2017b), which could promote preferential pH_i regulation. One particularly interesting study would be to see if *P. pardalis* are capable of pH_e regulation if the optimal conditions are provided as their capacity for pH_e compensation is extremely limited in their natural environment.

6.4.5 Mechanism(s) of preferential pH_i regulation

Lastly, one area that needs to be addressed is the molecular and cellular mechanisms of preferential pH_i regulation as nothing is presently known. This may be a challenging task given that different tissues have different responses (Shartau et al., 2017a), that there are differences between developmental stages (Shartau et al., 2016b), all of which may use different mechanisms. Additionally, while Huynh et al. indicated that cell culture approach may work for investigating the mechanism of preferential pH_i regulation, they also showed that the *in vitro* response differs from the *in vivo* response; possibly suggesting that extrinsic factors are involved (Huynh et al., 2011a). However, as preferential pH_i regulation did occur *in vitro*, this may represent one possible approach. Another possible technique is to use tissue slices (e.g. liver) exposed to hypercarbia and treated with various pharmacological inhibitors to assess how pH_i changes. This technique leaves cells in their extracellular matrix, allowing them to associate with each other and may be more natural than cell culture; this approach has been widely used in toxicology work in salmonids (Lemaire et al., 2011; Singh et al., 1996; Thohan et al., 2001). Regardless of the approach used, understanding the mechanisms will be important to fully understand how preferential pH_i is regulated and how it functions in response to different types of acid-base disturbances and in different environments.

6.5 Summary and final thoughts

At the start of this dissertation, coupled pH regulation had been considered 'the' pattern of acid-base regulation for decades (Albers, 1970; Cameron, 1989b; Heisler, 1984; Occhipinti and Boron, 2015; Roos and Boron, 1981) and preferential pH_i regulation was a novel and rare strategy of acid-base regulation limited to a mere four species: three fishes and one aquatic tetrapod. This dissertation provides evidence that preferential pH_i regulation is no longer a novel, nor rare strategy of acid-base regulation and thus may represent a paradigm shift regarding vertebrate acid-base regulation. Unexpectedly, preferential pH_i regulation does not confer uniform protection for tissues against all types of acid-base disturbances, at least in white sturgeon (Chapter 2). However, my findings indicate that adults of at least an additional 15 species (Chapter 3) and embryos of two species (Chapters 4 and 5), use preferential pH_i regulation during severe acute hypercarbia, which is an exciting expansion in the number of species exhibiting this strategy. The most interesting and surprising finding in this dissertation is that developing amniotes use preferential pH_i regulation (Chapters 4 and 5). The work in Chapters 3-5 required the original hypothesis of my thesis, that preferential pH_i regulation evolved in the ancestors of the basal euteleostomi, to be modified to: preferential pH_i regulation is an embryonic strategy that is either retained or lost in adults. The implication of this, which remains to be fully tested, is that all vertebrates use preferential pH_i regulation at one point during their life history. The putative retention of this embryonic strategy of acid-base regulation may have been an exaptation for maintaining pH homeostasis as adults in challenging environments, including during the major evolutionary transition to air breathing and the transition from life in water to life on land.

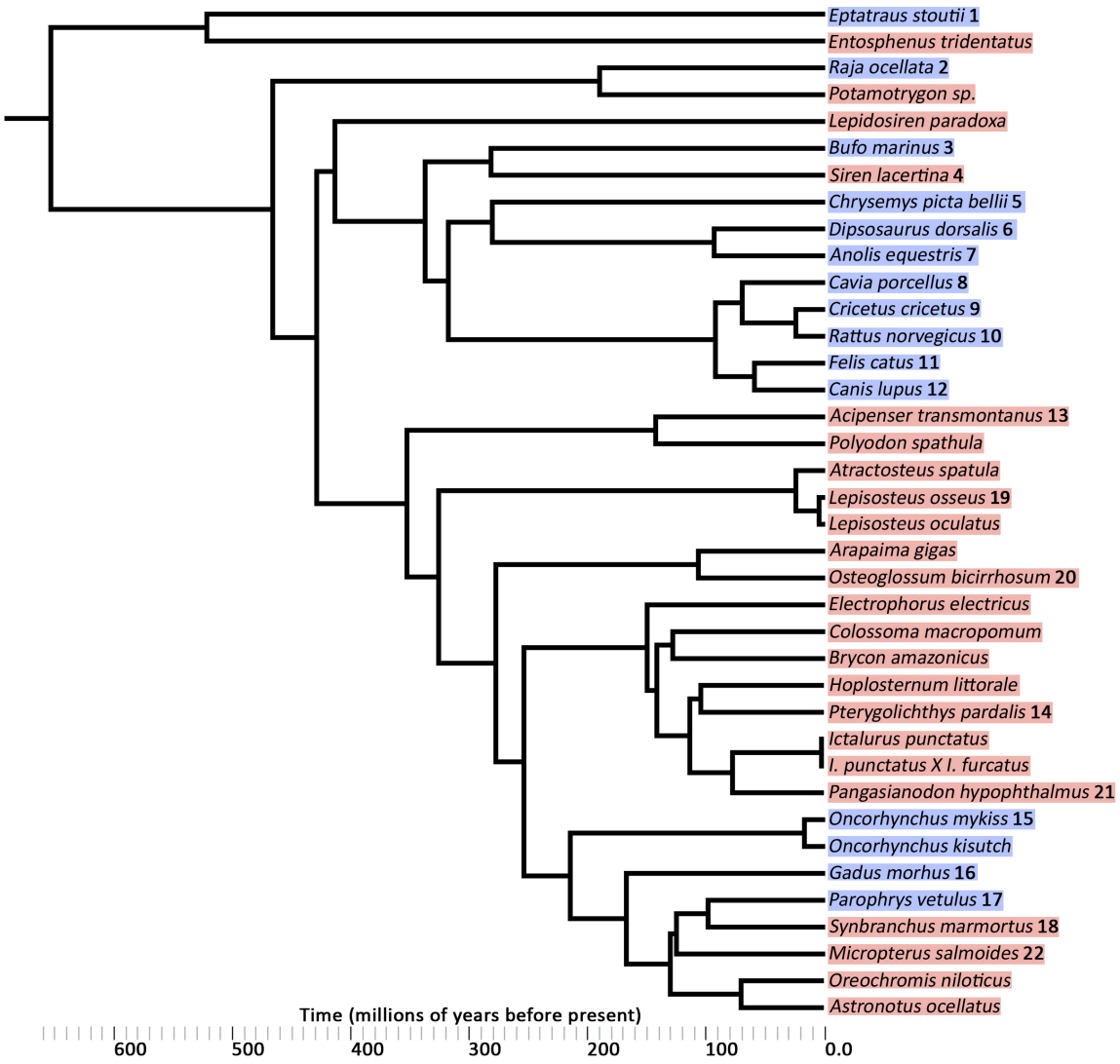

Figure 6.1: Phylogeny showing the distribution of preferential intracellular pH (pH_i) regulation and coupled pH regulation amongst vertebrates when exposed to acute >2 kPa PCO_2 following completion of dissertation research. Species using preferential pH_i regulation during severe acute hypercarbia are indicated in pink, while those using coupled pH regulation are indicated in blue. This phylogeny builds on Figure 1.4 and includes species examined prior to, and during this dissertation. All species were examined during Chapter 3 with the exception of *L. osseus*, *O. bicirrhosum*, *P. hypophthalmus*, and *M. salmoides*. Data from *L. osseus*[19], *O. bicirrhosum*[20], and *M. salmoides*[22] is not included in this thesis due to limited n's from sampling; however, based on the CO_2 tolerance assay, they preferentially regulate pH_i and are included in this figure to further demonstrate the prevalence of preferential pH_i regulation. Results from *P. hypophthalmus* are part of an unpublished project 21(R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong, M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong, C.J.B., unpublished). Other relevant references are indicated by numbers following

species name - 1(Baker et al., 2015), 2(Wood et al., 1990), 3(Snyder and Nestler, 1991), 4(Heisler et al., 1982), 5(Wasser et al., 1991), 6(Snyder et al., 1995), 7(Snyder et al., 1995), 8(Malan et al., 1985), 9(Wood and Schaefer, 1978), 10(Gonzalez and Clancy, 1986a), 11(Yaksh and Anderson, 1987), 12(Arieff et al., 1976), 13(Baker et al., 2009a), 14(Brauner et al., 2004), 15(Wood and LeMoigne, 1991), 16(Larsen et al., 1997), 17(Wright et al., 1988), 18(Heisler, 1982). Phylogenetic relationships are based on (2009) and branch lengths are taken from various references utilizing fossil and molecular estimates of divergence times (Aschliman et al., 2012; Betancur-R et al., 2013; Betancur-R et al., 2015; Blair, 2005; Macqueen and Johnston, 2014; Meredith et al., 2011; Zhang et al., 2013); the phylogenetic tree was created using Mesquite (Maddison and Maddison, 2017).

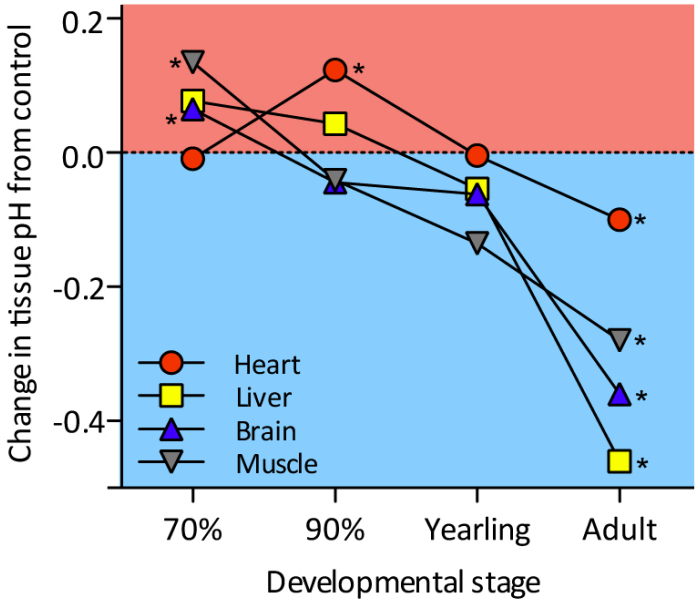

Figure 6.2: Difference in tissue pH during development in turtles. Difference in tissue pH (pH_i) during development is shown following exposure to hypercarbia relative to normocarbia in *Chelydra serpentina* [common snapping turtle; at 70% and 90% to hatch and in yearlings] and adult *Chrysemys picta bellii* (western painted turtles). Control pH values for pH_i of heart (red circle), liver (yellow square), brain (blue triangle) and muscle (grey inverse triangle) were subtracted from the values determined following either 1 h exposure to 13 kPa PCO_2, 9 kPa PO_2 in *C. serpentina* or 1 h exposure to 6.5 kPa arterial PCO_2 in *C. picta bellii*. Values ≥ 0 in the light red portion of the figure are indicative of preferential pH_i regulation while values ≤ in the light blue portion of the figure are indicative of coupled pH regulation. This figure shows that turtles preferentially regulate pH_i early in development and that the capacity for pH_i regulation is reduced throughout development. Significant changes in pH_i from control are indicated by asterisk ($P<0.05$); in all developmental stages extracellular pH (pH_e) was significantly reduced during hypercarbia exposure ($P<0.05$).

Table 6.1: Fish species investigated in this dissertation.

Order	Family	Species	Pattern of acid-base regulation	Biogeographical realm[1]	Water/air breather
Petromyzontiformes	Petromyzontidae	*Entosphenus tridentatus*	ppHi	Nearctic	Water
Myliobatiformes	Potamotrygonidae	*Potamotrygon sp.*	ppHi	Neotropic	Water
Ceratodontiformes	Lepidosirenidae	*Lepidosiren paradoxa*	ppHi	Neotropic	Air
Acipenseriformes	Polydontidae	*Polyodon spathula*	ppHi	Nearctic	Water
	Acipenseridae	*Acipenser transmontanus*[2,3]	ppHi	Nearctic	Water
Leisosteiformes	Lepisosteidae	*Lepisosteus oculatus*	ppHi	Nearctic	Air
		Atractosteus spatula	ppHi	Nearctic	Air
Osteoglossiformes	Osteoglossidae	*Arapaima gigas*	ppHi	Neotropic	Air
Gymnotiformes	Gymnotidae	*Electrophorus electricus*	ppHi	Neotropic	Air
Characiformes	Serrasalmidae	*Colossoma macropomum*	ppHi	Neotropic	Water
	Characidae	*Brycon amazonicus*	ppHi	Neotropic	Water
Siluriformes	Callichthyidae	*Hoplosternum littorale*	ppHi	Neotropic	Air
	Loricariidae	*Pterygoplichthys pardalis*[2,4]	ppHi	Neotropic	Air
	Ictaluridae	*Ictalurus punctatus*	ppHi	Nearctic	Water
		I. punctatus X *I. furcatus*	ppHi	Nearctic	Water
	Pangasiidae	*Pangasianodon hypophthalmus*[5]	ppHi	Indomalayan	Air

Order	Family	Species	Pattern of acid-base regulation	Biogeographical realm[1]	Water/air breather
Salmoniformes	Salmonidae	*Oncorhynchus kisutch*	Coupled	Nearctic	Water
		Oncorhynchus mykiss[2,6]	Coupled	Nearctic	Water
Synbranchiformes	Synbranchidae	*Synbranchus marmoratus*[2,7]	ppHi	Neotropic	Air
Perciformes	Cichlidae	*Oreochromis niloticus*	ppHi	Africotropical	Water
		Astronotus ocellatus	ppHi	Neotropic	Water

Taxonomic information for order and family are indicated, pattern of acid-base regulation (preferential pH_i regulation – ppHi, or coupled pH regulation – coupled) as determined from Chapter 3 results are shown. Biogeographical realms are indicated in order to show that the survey included fishes from various regions. A mix of water and air breathers were used, this is indicated. All fishes were primarily freshwater or freshwater-brackish inhabitants. All fishes were examined as part of Chapter 3 unless otherwise stated, footnotes indicate applicable references.[1](Udvardy, 1975), [2]Chapter 3, [3](Baker et al., 2009a), [4](Brauner et al., 2004), [5](R.B.S., M. Sackville, C. Damsgaard, L.M. Phuong, M. Hvas, T. Wang, M. Bayley, D.T.T. Huong, N.T. Phuong, C.J.B., unpublished), [6](Wood and LeMoigne, 1991), [7](Heisler, 1982).

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Appendix: A note on the methodology of pH measurements

The majority of this thesis relies on measurements of pH_e and pH_i to determine the pattern of acid-base regulation in the animals examined; consequently, reliable and accurate pH measurements are critical to the work and conclusions of my thesis.

Extracellular pH measurement

In Chapters 2 and 3, pH_e measurements were obtained from blood taken via caudal puncture which may differ from that of dorsal aortic blood drawn from a cannulated fish as performing cannulations was not always possible. To avoid the negative effects associated with caudal puncture for blood sampling, prior to sampling I lightly anesthetized fish in their container while ensuring sufficient aeration (either air or treatment gas); fish were then sampled in the container while keeping them submerged except for the site of the caudal puncture. This ensured the fish did not struggle, nor became hypoxic during sampling. To ensure pH values obtained via caudal puncture produced pH values comparable to those taken via dorsal aorta cannulation, I compared the two techniques in rainbow trout. pH_e from dorsal aorta cannulated and caudal puncture in control fish were 7.93 ± 0.03 (mean ± s.e.m.; n=12) and 7.83 ± 0.04 (n=8) pH units, respectively. In fish exposed to 1.5 kPa PCO_2 for 24 h, dorsal aorta cannulated fish and caudal puncture were 7.53 ± 0.04 (n=4) and 7.41 ± 0.04 (n=7) pH units, respectively. The pH_e differences between sampling technique for control and 1.5 kPa PCO_2 are not significant (independent samples t-test, $P>0.05$) and the relative difference between techniques is similar (0.101 and 0.122 pH units, in fish sampled via cannulation and caudal puncture, respectively). Differences between techniques can be largely attributed to stress during caudal puncture sampling, and some mixing of arterial and venous blood where the latter may be slightly lower than arterial pH_e in trout (Eddy, 1976). Despite caudal puncture sampling underestimating pH_e, through careful technique, this difference was minimized and the relative differences between control and treatment are still maintained; thus, use of caudal puncture provides a quick and reliable method of blood sampling.

Intracellular pH measurement

To assess pH_i regulation in response to acid-base challenges, I measured pH_i using the metabolic inhibitor tissue homogenate (MITH) method. The MITH method was first described and validated by Portner et al. (Portner et al., 1990) and later validated for use at high CO_2 by Baker et al. (2009b). The MITH method utilizes a simple protocol, described here. First, the tissues are quickly excised from the animal, ideally in <2 minutes and placed in aluminum foil or Eppendorf tubes, and immediately placed in liquid nitrogen. Tissues can then be transferred for storage at -80^{o}C for up to three months as validated by Baker et al. (2009b) (see below for more detail). Tissue pH is measured by grinding tissue into a fine powder under liquid nitrogen, which is then transferred to an Eppendorf tube containing metabolic inhibitor (KF [150 mmol/l], Na_2NTA [6 mmol/l]; exact concentrations of KF and Na_2NTA may vary depending on intracellular concentration of K and Na) at a ratio of 1:5 – 1:10 tissue:metabolic inhibitor, and gently vortexed. Finally, pH_i is measured on this supernatant using a pH electrode (Radiometer Analytical SAS pH electrode; CK2401C, Cedex, France) thermostated to the temperature at which the fish had been held.

The MITH method of measuring pH_i has previously been found to provide accurate, repeatable measurements of pH_i in several tissues from worms (*Sipunculus nudas*), squid (*Illex illecebrosus*), trout (*Oncorhynchus mykiss*), toads (*Bufo marinus*), and rats (Portner et al., 1990). This method of measuring pH_i was found to provide comparable pH_i values, with less variability compared to the older and commonly used dimethyloxazolidinedione (DMO) technique which is more variable and has considerable time delay to reach equilibrium in the tissue. The latter can vary from <30 s to ~ 1 h (Portner et al., 1990) while tissues can be dissected out immediately using the MITH technique. The MITH technique has been used in fish (Baker and Brauner, 2012; Baker et al., 2015; Brauner et al., 2004; Regan et al., 2016) and non-fish (Busk et al., 1997; Galli and Richards, 2012; Portner et al., 1990) studies.

Use of the MITH technique has been validated for both storage duration and measurement of pH_i from tissues exposed to high CO_2 tensions (Baker et al. 2009b). Baker et al. (2009b) validated the use of the MITH method on sturgeon red blood cell pH_i measurements following CO_2 exposure of up to 10 kPa PCO_2 with storage in either liquid nitrogen or -80^{o}C for 90 days. They observed no differences in pH_i of red blood cells measured immediately or following 30 days of storage at -80^{o}C. Additionally, the pH_i values obtained from the MITH

method were identical to those obtained using the freeze-thaw method (Zeidler and Kim, 1977), which involves repetitively freezing and thawing red blood cells and does not use any chemicals.

These storage durations and procedures using the MITH procedure have been used numerous times in fish (Baker and Brauner, 2012; Baker et al., 2015; Brauner et al., 2004; Regan et al., 2016) and non-fish studies, including the freshwater turtle (*Trachemys scripta*; (Galli and Richards, 2012), *Rana catesbeiana* tadpoles (Busk et al., 1997), and as indicated above, in worms, squid, toads, and rats (Portner et al., 1990) during CO_2 exposure ranging from normocarbia to 12 kPa PCO_2.