

COMMENTARY

Preferential intracellular pH regulation: hypotheses and perspectives

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ABSTRACT

The regulation of vertebrate acid–base balance during acute episodes of elevated internal P_{CO_2} is typically characterized by extracellular pH (pH_e) regulation. Changes in pH_e are associated with qualitatively similar changes in intracellular tissue pH (pH_i) as the two are typically coupled, referred to as ‘coupled pH regulation’. However, not all vertebrates rely on coupled pH regulation; instead, some preferentially regulate pH_i against severe and maintained reductions in pH_e . Preferential pH_i regulation has been identified in several adult fish species and an aquatic amphibian, but never in adult amniotes. Recently, common snapping turtles were observed to preferentially regulate pH_i during development; the pattern of acid–base regulation in these species shifts from preferential pH_i regulation in embryos to coupled pH regulation in adults. In this Commentary, we discuss the hypothesis that preferential pH_i regulation may be a general strategy employed by vertebrate embryos in order to maintain acid–base homeostasis during severe acute acid–base disturbances. In adult vertebrates, the retention or loss of preferential pH_i regulation may depend on selection pressures associated with the environment inhabited and/or the severity of acid–base regulatory challenges to which they are exposed. We also consider the idea that the retention of preferential pH_i regulation into adulthood may have been a key event in vertebrate evolution, with implications for the invasion of freshwater habitats, the evolution of air breathing and the transition of vertebrates from water to land.

KEY WORDS: Acid–base regulation, Preferential pH_i regulation, Fish, Amniotes, Hypercarbia, Development, Physiology

Introduction

Acid–base regulation is an essential physiological process that demands tight control. It is well known that absolute physiological pH values differ between species and 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, 1989; 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 (Occhipinti and Boron, 2015; Putnam and Roos, 1997). The degree to which a pH change affects function depends on the system in question.

Acid–base disturbances may arise from respiratory or metabolic sources (see Glossary). Respiratory acidoses occur as a result of an increase in blood CO_2 , either from the environment (hypercarbia; see Glossary) or by retention of metabolically produced CO_2 (hypercapnia; see Glossary); typical arterial values of partial pressure of CO_2 (P_{CO_2}) for adult water-breathing and bimodally breathing fishes, and 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 P_{CO_2} (Arieff et al., 1976; Malan et al., 1985; Wood and Schaefer, 1978; Yaksh and Anderson, 1987), respectively. Any increase in P_{CO_2} beyond those values shifts the equilibrium of the CO_2 hydration reaction ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$), promoting the formation of H^+ and HCO_3^- and lowering pH, resulting in acidosis. Metabolic acidoses occur because of the production of metabolically generated acid, for example, following exercise or hypoxia, as a result of anaerobic metabolism, which lowers HCO_3^- at a relatively constant P_{CO_2} (Occhipinti and Boron, 2015).

Typically, organismal acid–base regulation in vertebrates is characterized by regulation of blood extracellular pH (pH_e). During an acute respiratory acidosis, reductions in pH_e are associated with qualitatively similar reductions in intracellular tissue pH (pH_i). Following the onset of a respiratory acidosis, the recovery of pH_i is often more rapid than that of pH_e , but complete correction of pH_i generally requires pH_e compensation (see Glossary) of >50% (Baker et al., 2015; Larsen et al., 1997; Wood and LeMoigne, 1991; Wood et al., 1990; Wood and Schaefer, 1978), which we refer to here as ‘coupled pH regulation’ (Fig. 1A,C). Coupled regulation is thought to represent the normal response to an acute persistent acidosis both *in vivo* and *in vitro*. Some vertebrates, however, do not exhibit coupled pH regulation during a severe acute acidosis, but instead preferentially and rapidly regulate pH_i , despite maintained reductions in pH_e that can exceed 1 pH unit (Fig. 1B,D) (Baker et al., 2009; Brauner and Baker, 2009; Shartau and Brauner, 2014). Although all cells have the capacity for some degree of pH_i regulation (Occhipinti and Boron, 2015; Putnam and Roos, 1997; Vaughan-Jones et al., 2009), in animals that employ coupled pH regulation, cells cannot fully compensate for changes in pH_i during a large sustained reduction in pH_e .

Although vertebrates are the focus of this Commentary, it is worth noting that limited studies on invertebrates during acute severe hypercarbia suggest that invertebrate acid–base regulation may be characterized by coupled pH regulation, similar to 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*) (Pörtner et al., 1998) have been observed. Unlike vertebrates, there is no evidence of preferential pH_i regulation in invertebrates to date; however, given the dearth of

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List of symbols and abbreviations

pH_e	extracellular pH
pH_i	intracellular tissue pH
P_{CO_2}	partial pressure of CO_2
P_{O_2}	partial pressure of O_2
RBC	red blood cell

data, it is premature to ascribe a single strategy of acid–base regulation to this large and diverse group of animals.

Preferential pH_i regulation is a phenomenon that has been identified in only a few vertebrate species to date, and it represents a notably different pattern of acid–base regulation, involving complete correction of pH_i in the absence of pH_e recovery. Based on our recent findings (Shartau et al., 2016), we hypothesize that preferential pH_i regulation may represent the basal and embryonic pattern of acid–base regulation in vertebrates, providing extreme tolerance to severe acid–base challenges. It is possible that this exceptional pattern of acid–base regulation represents an exaptation (see Glossary) associated with both the evolution of air breathing and the transition of life from water to land – events that are likely to have posed challenges to acid–base homeostasis.

This Commentary will introduce readers to preferential pH_i regulation as a pattern of acid–base regulation used during acute acid–base challenges, and discuss how it differs from the more commonly accepted vertebrate pattern of coupled pH regulation. We will discuss the differences between these two means of controlling

Glossary**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 P_{CO_2} .

pH compensation

A return of pH to its normal value following an acid–base disturbance.

Respiratory acidosis

Reduced pH because of increased blood CO_2 from an environmental or internal source.

acid–base balance. Next, we will show that preferential pH_i regulation is more prevalent than previously thought by discussing the increasing number of species that display this trait, considering both embryonic and adult stages. Following that, we will discuss our hypothesis that preferential pH_i regulation is prevalent in embryonic vertebrates but is lost during the course of development in most species. Finally, ideas concerning the selective pressure for preferential pH_i regulation and its potential role in vertebrate evolution will be examined.

Coupled pH regulation

Findings from *in vivo* studies conducted on a relatively small selection of juvenile and adult mammals, reptiles, amphibians and fishes have established that vertebrates utilize coupled pH regulation during compensation for respiratory acid–base disturbances (Brauner and Baker, 2009; Cameron, 1989; Occhipinti and Boron, 2015; Shartau and Brauner, 2014). Likewise, *in vitro* cell culture studies have demonstrated that most vertebrate cells are unable to completely compensate for an acute respiratory acidosis while pH_e remains reduced, indicating that complete pH_i recovery is contingent on significant recovery of pH_e . Coupled pH regulation has formed the dogma of vertebrate acid–base homeostasis for decades (Albers, 1970; Cameron, 1989; Heisler, 1984; Occhipinti and Boron, 2015; Roos and Boron, 1981). Visual representation of coupled pH regulation (Fig. 1A,C) illustrates that as pH_e is reduced, pH_i falls in a qualitatively similar manner. Although the recovery of pH_i generally occurs more rapidly than that of pH_e , complete pH_i compensation has typically been associated with pH_e recovery of ~50% or greater (Baker et al., 2015; Larsen et al., 1997; Wood and LeMoigne, 1991; Wood et al., 1990; Wood and Schaefer, 1978). This limit to pH_i correction is in clear contrast to the recovery seen in vertebrates exhibiting preferential pH_i regulation. These species can tolerate severe reductions in pH_e with no change in pH_i – in some cases pH_i actually increases (Fig. 1B,D). The pattern of coupled pH regulation has been thoroughly described in a number of reviews (see Brauner and Baker, 2009; Cameron, 1989; Heisler, 1984; Occhipinti and Boron, 2015), so will only briefly be discussed here.

In vivo studies

Marine and freshwater fishes experiencing a respiratory acidosis that is due to acute hypercarbia exposure exhibit coupled pH regulation, which has been observed in agnathans (Baker et al., 2015), elasmobranchs (Heisler et al., 1988; Wood et al., 1990) and many teleosts (e.g. Brauner and Baker, 2009; Hayashi et al., 2004; Larsen et al., 1997; Wood and LeMoigne, 1991). Coupled pH regulation is

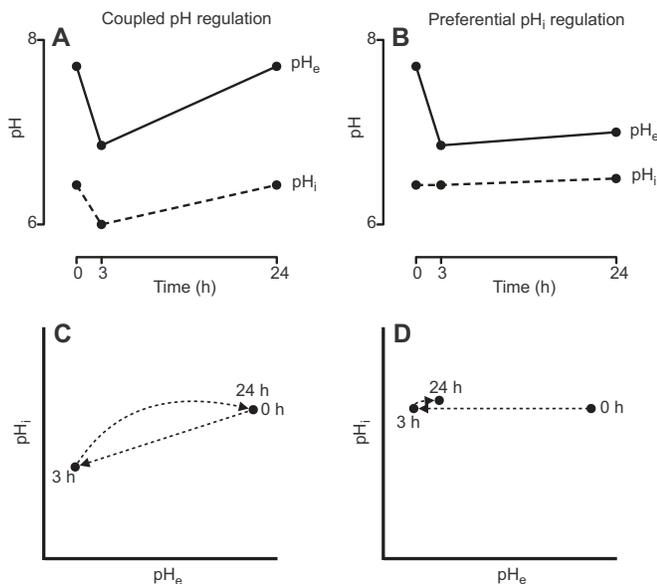


Fig. 1. Representation of the typical response of vertebrates during acute sustained hypercapnia utilizing either coupled pH regulation or preferential intracellular pH (pH_i) regulation. (A,C) In species utilizing coupled pH regulation, a hypercarbia-induced respiratory acidosis (initiated at $t=0$) leads to a rapid reduction in both extracellular pH (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 recovery depends on the severity of the pH depression and the ability for net acid excretion to the environment. pH_i often recovers more rapidly than pH_e , but complete pH_i compensation generally requires >50% of complete pH_e recovery. (B,D) 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.

driven primarily by acid extrusion and base uptake at the gills; although the kidneys are the primary site of acid–base regulation in air breathers, they probably play only a minor role in acid–base exchange in water breathers but are important for base retention (Brauner and Baker, 2009; Cameron, 1989). In fishes that exhibit coupled pH regulation, compensation of pH_e and pH_i occurs over the initial 24–72 h during sustained hypercarbia exposure (Fig. 1A,C), with pH_i recovery usually preceding that of pH_e , partly because intracellular fluids typically display a lower pH than the extracellular blood environment, which places the pK' (the apparent dissociation constant) of the $\text{CO}_2\text{--HCO}_3^-$ reaction closer to pH_i . Thus, relatively less HCO_3^- is required to compensate pH_i compared with 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; Ultsch, 1996).

Coupled pH regulation during acute hypercarbia is accomplished by a net increase in plasma HCO_3^- associated with an equimolar loss of Cl^- . The extent of this HCO_3^- elevation, however, appears to be limited, in that plasma $[\text{HCO}_3^-]$ rarely exceeds 27–33 mmol l^{-1} during exposure to acute hypercapnia – this has been previously referred to as the ‘apparent bicarbonate concentration threshold’ (Heisler, 1984). This threshold is associated with a ceiling of complete pH_e compensation in most fish during acute exposure to CO_2 tensions below 2–2.5 kPa P_{CO_2} (Baker et al., 2009; Brauner and Baker, 2009). 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 (see Glossary; Baker et al., 2015). The osmo- and iono-conforming Pacific hagfish (*Eptatretus stoutii*), a coupled pH regulator, has a plasma $[\text{Cl}^-]$ of $\sim 458 \text{ mmol l}^{-1}$, which is three to four times higher than that of typical teleosts, and perhaps as a result, hagfish are able to increase plasma $[\text{HCO}_3^-]$ to $>80 \text{ mmol l}^{-1}$, driving pH_e and pH_i recovery during exposure to severe hypercarbia (P_{CO_2} of $\sim 6.5 \text{ kPa}$) (Baker et al., 2015). In contrast to acute hypercarbia, chronic CO_2 exposure allows some teleosts to elevate $[\text{HCO}_3^-]$ well beyond this threshold, aiding pH_e recovery. Rainbow trout (*Oncorhynchus mykiss*) subjected to increasing hypercarbia over 3 days to reach 3.47 kPa P_{CO_2} , and maintained at this level for an additional 3 days, had a blood $[\text{HCO}_3^-]$ of 66 mmol l^{-1} (Dimberg, 1988). Similarly, European eels (*Anguilla anguilla*) gradually exposed to and maintained at 6 kPa P_{CO_2} for 6 weeks had plasma $[\text{HCO}_3^-]$ of 73 mmol l^{-1} (McKenzie et al., 2003). The degree and speed of pH_e compensation in aquatic coupled pH regulators during acute and chronic hypercarbia exposure 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 adult air-breathing tetrapods, coupled pH regulation is observed in all taxa where pH_e and pH_i have been measured during an acute respiratory acidosis; furthermore, there is no evidence to date for preferential pH_i regulation in these adult animals. 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 P_{CO_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*) for up to 6 h during a severe acute respiratory acidosis associated with diving (Wasser et al., 1991) (see Table 1 for further detail). Coupled pH regulation also occurs during an acute metabolic acidosis following exhaustive exercise in the saltwater crocodile (*Crocodylus porosus*) (Baldwin et al., 1995), strongly supporting

speculation that coupled pH regulation is a general pattern for all pH disturbances in adult air-breathing vertebrates. Further support for this conclusion is the substantial evidence that mammals subjected to an acute respiratory acidosis display coupled pH regulation. Adult dog (Arieff et al., 1976) and cat (Yaksh and Anderson, 1987) pH_e and pH_i were reduced following exposure to $\geq 8 \text{ kPa } P_{\text{CO}_2}$ for 3 h and 10 min, respectively. In guinea pigs exposed to 15 kPa P_{CO_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 also been corroborated in rats (Gonzalez and Clancy, 1986; Litt et al., 1985) and hamsters (Malan et al., 1985) during acute hypercarbia exposure (Table 1). 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 recovery occurs through coupled pH regulation, as has been observed for a relatively small number of fish species that have been examined to date.

In vitro studies

There are many cell culture studies that have examined pH_i regulation in cells following transitory reductions in pH_e (e.g. 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 of these have been conducted in the presence of sustained and elevated CO_2 ; however, 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 is that (1) reducing pH_e causes pH_i to be reduced and (2) complete recovery of pH_i 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 $[\text{HCO}_3^-]$ 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 acute pH_i reduction when pH_e is reduced in a cell culture environment, characteristic of coupled pH regulation. Of course, pH_i responses recorded *in vitro* may not necessarily be representative of patterns of whole-animal acid–base regulation; however, in white sturgeon exposed to hypercarbia, the pH_i protective response observed in liver tissue *in vivo* (Baker et al., 2009) was also observed to some degree in isolated hepatocytes exposed to hypercarbia that reduced pH_e to a similar magnitude (Huynh et al., 2011a).

Preferential pH_i regulation in adult vertebrates

Adult fishes make up the majority of species where preferential pH_i regulation has been identified thus far. The white sturgeon (an acipenseriform) is presently the most basal actinopterygian in which preferential pH_i regulation has been observed (Fig. 2). Exposure of white sturgeon for 48 h to 6 kPa P_{CO_2} reduced pH_e by $\sim 0.6 \text{ pH}$ units, but no reductions in pH_i were observed in heart, liver, brain or white muscle (Baker et al., 2009); following 6 h of exposure to 12 kPa P_{CO_2} , pH_e fell by $\sim 1.0 \text{ pH}$ unit, but liver pH_i increased by

Table 1. Examples of the changes in blood and tissue pH (pH_e and pH_i, respectively) following a severe acute hypercapnia-induced respiratory acidosis in selected vertebrates

Species	Source of acidosis	P _{CO₂} (kPa)	Sampling time (h)	pH _e change	Tissue	pH _i change	References
Rat (<i>Rattus norvegicus</i>)	Hypercarbia	7–8	4	↓	Heart Muscle	↓ ↓	Gonzalez and Clancy, 1986
Hamster (<i>Cricetus cricetus</i>)	Hypercarbia	70	0.25	↓	Brain	↓	Litt et al., 1985
	Hypercarbia	12	1.5	↓	Brain	↓	Malan et al., 1985
					Liver Heart Muscle	↓ ↓ ↓	
Guinea pig (<i>Cavia porcellus</i>)	Hypercarbia	15	2 and 8	↓	Lung Kidney Heart Muscle	↓ ↓ ↓ ↓	Wood and Schaefer, 1978
Dog (<i>Canis lupus familiaris</i>)	Hypercarbia	8–14	1	↓	Brain Muscle	↓ ↓	Arieff et al., 1976
Cat (<i>Felis catus</i>)	Hypercarbia	8	0.17	↓	Brain	↓	Yaksh and Anderson, 1987
Common snapping turtle (<i>Chelydra serpentina</i>) 70% to hatch	Hypercarbia +hypoxia (9 kPa P _{O₂})	13	1	↓	Heart	↔	Shartau et al., 2016
					Brain	↑	
90% to hatch	Hypercarbia +hypoxia (9 kPa P _{O₂})	13	1	↓	Liver	↔	Shartau et al., 2016
					Lung	↑	
					Kidney	↔	
					Muscle	↑	
					Heart	↑	
Yearling	Hypercarbia +hypoxia (9 kPa P _{O₂})	13	1	↓	Brain	↔	Shartau et al., 2016
					Liver	↔	
					Lung	↔	
					Kidney	↔	
					Muscle	↔	
Western painted turtle (<i>Chrysemys picta bellii</i>)	Submergence	~6.5	1	↓	Heart	↓	Wasser et al., 1991
					Liver	↓	
					Brain	↓	
					Muscle	↓	
Knight anole (<i>Anolis equestris</i>)	Hypercarbia	5	1	↓	Brain	↓	Snyder et al., 1995
					Muscle	↓	
Desert iguana (<i>Dipsosaurus dorsalis</i>)	Hypercarbia	5	1	↓	Brain	↓	Snyder et al., 1995
					Muscle	↓	
Cane toad (<i>Bufo marinus</i>)	Hypercarbia	5	1	↓	Muscle	↓	Snyder and Nestler, 1991; Toews and Heisler, 1982
					Heart	↓	
					Brain	↓	
					Liver	↓	
					Skin	↓	
Greater siren (<i>Siren lacertina</i>)	Hypercarbia	6.3	72–80	↓	Heart	↔	Heisler et al., 1982
					Muscle	↔	
American bullfrog (<i>Rana catesbeiana</i>) tadpoles	Hypercarbia	5	2	↓	Liver	↓	Busk et al., 1997
					Muscle	↓	
Pacific hagfish (<i>Eptatretus stoutii</i>)	Hypercarbia	6	3	↓	RBC	↓	Baker et al., 2015
					Heart	↓	
					Liver	↓	
					Muscle	↓	
Atlantic big skate (<i>Raja ocellata</i>)	Hypercarbia	1	0.5 and 2	↓	RBC	↓	Wood et al., 1990
					Heart	↓	
					Brain	↓	
					Muscle	↓	
						↓	
White sturgeon (<i>Acipenser transmontanus</i>)	Hypercarbia	6	3	↓	RBC	↓	Baker et al., 2009
					Heart	↑	
					Liver	↔	
					Brain	↑	
					Muscle	↔	
Armoured catfish (<i>Pterygoplichthys pardalis</i>)	Hypercarbia	4.3	3	↓	RBC	↓	(Brauner et al., 2004)
					Heart	↔	

Continued

Table 1. Continued

Species	Source of acidosis	P_{CO_2} (kPa)	Sampling time (h)	pH_e change	Tissue	pH_i change	References
Channel catfish (<i>Ictalurus punctatus</i>)	Hypercarbia	1.5	2	↓	Liver	↔	(Cameron, 1980)
					Muscle	↔	
Marbled swamp eel (<i>Synbranchus marmoratus</i>)	Air exposure	3.5	96	↓	Whole body	↓	(Heisler, 1982)
					Heart	↔	
Lemon sole (<i>Parophrys vetulus</i>)	Hypercarbia	1	0.5	↓	Muscle	↔	(Wright et al., 1988)
					RBC	↓	
Cod (<i>Gadus morhua</i>)	Hypercarbia	1	3	↓	Heart	↓	(Larsen et al., 1997)
					Liver	↔	
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Hyperoxia	~1	3	↓	Muscle	↓	(Wood and LeMoigne, 1991)
					RBC	↓	
					Brain	↓	
					Muscle	↓	
					Gill	↔	

Studies used adult animals unless indicated otherwise. P_{CO_2} tensions indicate the level of environmental CO_2 exposure, except in western painted turtle, marbled swamp eel and rainbow trout, where the values indicate arterial P_{CO_2} . RBC, red blood cell. ↓, pH decrease; ↔, no pH change; ↑, pH increase.

0.2 pH units (Baker and Brauner, 2012). Protection of pH_i during exposure to hypercarbia in white sturgeon appears to be nearly instantaneous. When heart pH_i was measured in real time at 2-min intervals using magnetic resonance imaging, there was no evidence for heart muscle pH_i ever decreasing (D. W. Baker, Physiological responses associated with aquatic hypercarbia in the CO_2 -tolerant white sturgeon *Acipenser transmontanus*, PhD thesis, University of British Columbia, 2010).

All other adult species in which preferential pH_i regulation has been observed are bimodal breathers. The armoured catfish (*Pterygoplichthys pardalis*), a tropical freshwater teleost, preferentially regulates pH_i of heart, brain, liver and white muscle during acid–base disturbances of different origins, including respiratory acidosis (hypercarbia) (Brauner et al., 2004), metabolic acidosis (exhaustive exercise and anoxia) and intravenous base addition (HCO_3^- injection) (Harter et al., 2014). Similarly, another tropical freshwater teleost, the marbled swamp eel (*Synbranchus marmoratus*), is able to preferentially regulate heart and white muscle pH_i during exposure to 3.5 kPa P_{CO_2} for 96 h, despite pH_e being reduced by ~0.6 pH units and remaining uncompensated throughout the exposure period (Heisler, 1982). Recently, preferential pH_i regulation has been observed in striped catfish (*Pangasianodon hypophthalmus*; 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), spotted gar (*Lepisosteus oculatus*), longnose gar (*L. osseus*) and alligator gar (*Atractosteus spatula*) (Shartau and Brauner, 2014) during severe acute hypercarbia. There is indirect evidence for preferential pH_i regulation in bowfin (*Amia calva*) (Brauner and Baker, 2009) and South American lungfish (*Lepidosiren paradoxa*) (Brauner and Baker, 2009; Sanchez et al., 2005; Shartau and Brauner, 2014), based upon the presence of severe uncompensated pH_e reductions, which are highly characteristic of preferential pH_i regulation; however, further studies are required to validate the pattern of acid–base regulation in these species.

To date, only one adult tetrapod is known to preferentially regulate pH_i – an aquatic bimodally breathing amphibian, the greater siren (*Siren lacertina*). Exposing the greater siren to aquatic hypercarbia of 6.3 kPa P_{CO_2} resulted in an uncompensated reduction in pH_e , but heart and white muscle pH_i were unchanged from values

recorded under normocapnic conditions following 32 h of exposure (Heisler et al., 1982), the earliest measure taken in that study. As it stands, the lack of evidence for preferential pH_i regulation in adult amniotes strongly suggests that these animals rely on coupled pH regulation (see previous section). Intriguingly, recent studies indicate that acid–base regulation in some embryonic amniotes may differ from that of adults – this is discussed in the next section.

Preferential pH_i regulation during development

Characterizing pH_i regulation in early development is challenging because of the difficulty of measuring pH_i in single cells *in vivo*; consequently, evidence supporting or refuting preferential pH_i regulation at early developmental stages is severely lacking. A number of studies have measured pH_i in early-stage embryos, demonstrating that, early in development, cells are capable of pH regulation just after fertilization and are able to compensate for an intracellular acid–base disturbance of almost 1 pH unit (FitzHarris and Baltz, 2009; Lane, 1999). Work on fish has also showed this pattern, as early-stage zebrafish embryos exposed to 3.3 kPa P_{CO_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). These studies indicate that during an acute acidosis, cells have the capacity to compensate pH_i 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 acidosis (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, but the embryos survive (Burggren et al., 2012; Mueller et al., 2014) – a pattern that is consistent with the pH_e changes observed in fish that preferentially regulate pH_i (Brauner and Baker, 2009). These studies did not

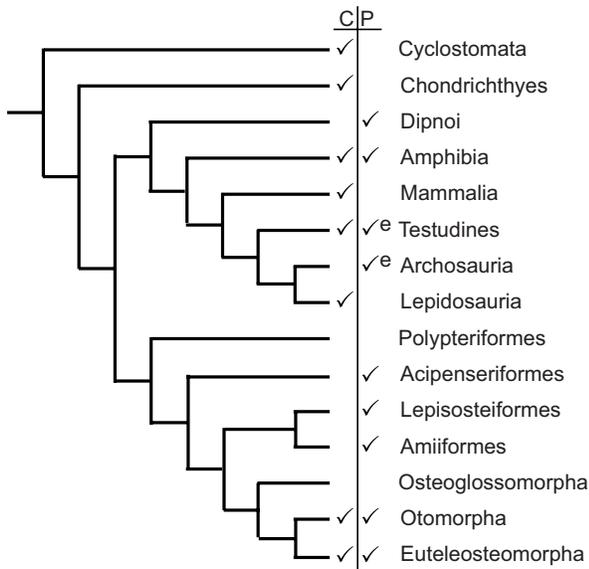


Fig. 2. Prevalence of preferential pH_i regulation amongst vertebrates exposed to an acute (<48 h) respiratory acidosis of ≥ 1 kPa blood P_{CO_2} . Groups containing species in which either coupled (C) or preferential (P) pH_i regulation has been observed in adults are indicated by ✓; embryos are indicated by ✓^e. Table 2 contains the information on the species in which this has been investigated, along with the relevant references.

measure pH_i ; however, based on these data, we hypothesize that embryonic chickens may preferentially regulate pH_i , making this a potential avian model that is clearly worthy of study.

The only simultaneous measurements of pH_e and pH_i that have been made in later-stage developing vertebrate embryos during acute acid–base disturbances were in the common snapping turtle (*Chelydra serpentina*) (Shartau et al., 2016) and American alligator (*Alligator mississippiensis*) (R.B.S., D.A.C., Z. F. Kohl, R. M. Elsey and C.J.B., unpublished observations), where preferential pH_i regulation was observed for the first time in amniotes. Turtles at three developmental stages (two embryonic and one post-hatch stage) were exposed to hypercarbic hypoxia (13 kPa P_{CO_2} , 9 kPa P_{O_2}) for 1 h to create a severe acute respiratory metabolic acidosis, similar to the above studies on embryonic chickens. It was observed that the pattern of acid–base regulation changes throughout development. Younger turtle embryos appeared to exhibit a more robust capacity for preferential pH_i regulation than turtles at post-hatch stages. Turtle embryos at 70% to hatch displayed a pH_e reduction of ~ 0.3 pH units, while pH_i increased in brain, lung and white muscle; no changes in pH_i were observed in heart, liver or kidney (Fig. 3). Embryos at 90% to hatch experienced a similar pH_e reduction, accompanied by an increase in heart pH_i , with no significant reductions in pH_i of the other tissues. Post-hatch yearling turtles exhibited a slightly smaller pH_e reduction (~ 0.25 pH units) and also displayed no significant decreases in pH_i of any tissue (Fig. 3) (Shartau et al., 2016). By our definition, all three of these stages exhibit preferential pH_i regulation (see Fig. 1), but interestingly, as development proceeded, there were fewer tissues in which pH_i was regulated above control values, which suggests a reduced capacity for preferential pH_i regulation later in development. This would potentially culminate with coupled pH regulation in adults, which is supported by data showing that pH_i in adult western painted turtles closely follows pH_e reductions during hypercapnia associated with diving (Fig. 3) (Wasser et al., 1991).

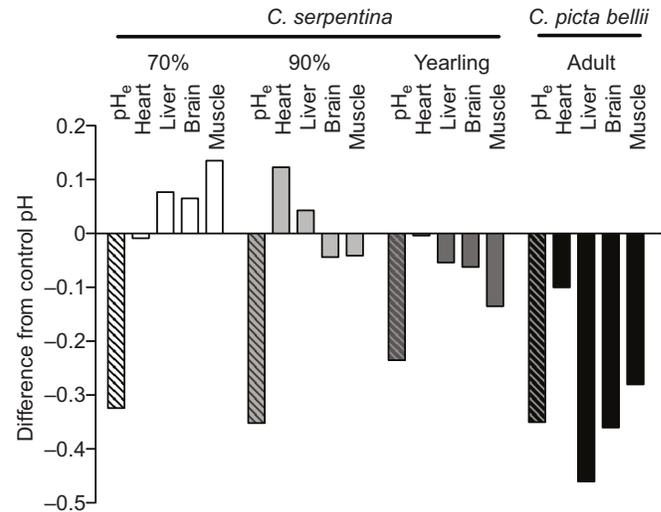


Fig. 3. Difference in blood and tissue pH during development in turtles. Difference in blood pH (pH_e) and tissue pH (pH_i) during development is shown following exposure to hypercarbia relative to normocarbica in common snapping turtles [*Chelydra serpentina*; at 70% (open bars) and 90% (shaded bars) to hatch, and in yearlings (dark shaded bars) (Shartau et al., 2016)] and adult western painted turtles [*Chrysemys picta bellii*, black bars (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 exposure to 13 kPa P_{CO_2} , 9 kPa P_{O_2} in common snapping turtles or 1 h exposure to 6.5 kPa arterial P_{CO_2} in western painted turtles. A value ≥ 0 is indicative of preferential pH_i regulation. Bars for pH_e are hatched.

Similarly, when American alligator embryos at 70% to hatch were exposed to 1 h hypercarbic hypoxia (13 kPa P_{CO_2} , 9 kPa P_{O_2}), they preferentially regulated pH_i . A pH_e reduction of ~ 0.4 pH units corresponded to significant increases in heart and brain pH_i ; no pH_i changes occurred in other tissues (i.e. no acidification; R.B.S., D.A.C., Z. F. Kohl, R. M. Elsey and C.J.B., unpublished observations). These findings indicate that embryonic American alligators also preferentially regulate pH_i at this stage of development and thus utilize a different acid–base regulatory strategy than adults, consistent with turtles, as described above. Although snapping turtle and American alligator embryos preferentially regulate pH_i during hypercarbia hypoxia, other studies observing preferential pH_i regulation in adult fishes have used hypercarbia alone. The response to hypercarbia alone in amniote embryos is unknown; consequently, the possible differences that are due to the effects of hypoxia on pH_e and pH_i during hypercarbia should be considered.

It is intriguing to hypothesize that vertebrate embryos in general may rely on preferential pH_i regulation to protect pH_i against sustained extracellular acid–base changes and that this ability is lost at some point post-hatch in amniotes and in certain amphibians and fishes, where coupled pH regulation becomes the dominant strategy for acid–base regulation. Clearly, more work needs to be conducted on a range of embryonic vertebrates during exposure to hypercarbia, as well as other acid–base disturbances, to determine whether preferential pH_i regulation is a general strategy of acid–base regulation during embryonic development.

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

As mentioned above, we hypothesize that preferential pH_i regulation may represent the basal pattern of acid–base regulation in vertebrates as a strategy during development, with adults either

retaining or losing this trait. The mechanisms responsible for preferential pH_i regulation are unknown, but allow embryos to compensate for acid–base challenges to pH_i , despite the incomplete formation in embryos of the extracellular compartment and associated structures that are required for coupled pH regulation. These challenges apply to all embryos, and acid–base challenges may arise from hypoxia and hypercarbia or increased metabolic activity throughout development (Tazawa et al., 1983). In the specific examples described above, additional acid–base challenges may be experienced in the form of hypoxia or hypercarbia, conditions associated with nests or egg masses (Booth, 1998; Grigg et al., 2010; Lutz and Dunbar-Cooper, 1984). Furthermore, embryos encapsulated within extra-embryonic structures (e.g. eggshells) face additional restrictions on the diffusion of O_2 and CO_2 (Goldberg et al., 2008; Tazawa, 1980), which makes the exchange of gases and acid–base equivalents with the environment challenging and limits the ability of the embryo to undergo coupled pH regulation (Erasmus et al., 1971).

Although turtles and alligators appear to lose the capacity for preferential pH_i regulation as adults, a number of adult fishes and an aquatic salamander preferentially regulate pH_i (Fig. 2, Table 2), perhaps retaining this ability from embryonic development (although developmental studies are largely lacking and clearly remain worthy of investigation). This retention of preferential pH_i regulation may be correlated with environmental conditions. For example, fish may lack the ability for coupled pH regulation because of the limited availability of acid–base equivalents in the water they inhabit. Tropical freshwater environments that are high in CO_2 and ion-poor, such as the Amazon River, where water P_{CO_2} can reach 8 kPa during diurnal fluctuations (Brauner et al., 2004; Heisler, 1984), may pose challenges for acid–base regulation and compensation through coupled pH regulation. Consequently, retention of preferential pH_i regulation in adults could play a crucial role in acid–base regulation in tropical freshwater fishes; indeed, a number of fish species known to preferentially regulate pH_i are found in the Amazon. It is possible that the first vertebrates to retain preferential pH_i regulation as adults included species making the transition from ion-rich environments (e.g. marine waters) to ion-poor environments (e.g. tropical freshwaters) – similar to current tropical systems such as the Amazon Basin – where their capacity for coupled pH regulation was diminished and thus preferential pH_i regulation may have protected pH_i against severe acidoses, including those induced by high CO_2 (Brauner and Baker, 2009; Shartau and Brauner, 2014). Vertebrates in ion-rich environments are likely to be able to use coupled pH regulation as there are sufficient acid–base equivalents for pH_e compensation during severe acidoses, including those resulting from high CO_2 ; this is observed in a number of marine species exposed to severe hypercarbia, including hagfish (Baker et al., 2015) and teleosts (Hayashi et al., 2004).

In adult bimodally breathing fishes, the retention of preferential pH_i regulation may provide a means of coping with acid–base challenges associated with air breathing (Shartau and Brauner, 2014). These fishes are typically capable of O_2 uptake from water or air, but excrete the majority of CO_2 to the water. Consequently, in bimodally breathing fishes, air breathing may lead to a rapid increase in blood P_{CO_2} , as CO_2 excretion rates at the gills are reduced as a result of emersion or reduced gill blood flow and/or gill ventilation. Depending on the species and conditions, blood P_{CO_2} can increase to >3 kPa during an air-breathing episode (Shartau and Brauner, 2014). All fish species (with the exception of one, the white sturgeon) currently known to use preferential pH_i

regulation as adults are bimodal breathers. Air-breathing in fishes is thought to have evolved in more tropical environments that likely experience both hypoxia and hypercapnia; 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 air breathing (Brauner and Baker, 2009; Shartau and Brauner, 2014).

We hypothesize that preferential pH_i regulation may have been an exaptation associated with niche expansion from water to land in the vertebrate lineage. This pattern of acid–base regulation possibly allowed these transitioning vertebrates, which likely possessed acid–base characteristics similar to those of present-day water breathers (high pH_e , low arterial P_{CO_2} and low plasma $[\text{HCO}_3^-]$) and still relied on the gills for exchange of acid–base ion equivalents (Ultsch, 1996, 2012; Witzmann, 2015), to protect pH_i during terrestrial excursions. Gradually, coupled pH regulation may have replaced preferential pH_i regulation as the strategy to maintain whole-body pH as (1) these terrestrial excursions became longer, (2) the animals' blood acid–base status became more similar to that of terrestrial air breathers (higher arterial P_{CO_2} , higher plasma $[\text{HCO}_3^-]$ and lower pH_e) and (3) the kidneys replaced the gills as the dominant organ for acid–base regulation.

The retention of preferential pH_i regulation beyond development may be associated with survival during severe acute acid–base disturbances; however, this pattern of acid–base regulation is diminished and ultimately lost in many adult vertebrates. Why preferential pH_i regulation was not retained more broadly can only be speculated upon at this time. Although preferential pH_i regulation may be metabolically less costly than coupled pH regulation (Baker and Brauner, 2012) and protects cardiac performance during acute hypercarbia exposure (Baker et al., 2011; Hanson et al., 2009; Shartau et al., 2016), there may be unquantified long-term costs associated with preferential pH_i regulation. This premise remains to be investigated. Ultimately, the benefit of preferential pH_i regulation over coupled pH regulation is that vertebrate embryos and adults can completely protect pH_i even if pH_e reductions are uncompensated, as occurs during severe acute respiratory acidoses; limited data suggest that preferential pH_i regulation may function to protect pH_i during other types of acid–base disturbances (Harter et al., 2014; Shartau et al., 2016).

Conclusions and future perspectives

Preferential pH_i regulation is a strategy of acid–base regulation that allows vertebrates to maintain pH homeostasis during severe acute acid–base disturbances under conditions in which compensation through coupled pH regulation would be limited. It has been observed in embryos of two amniote species, as well as in several adult vertebrates (Fig. 2). Based on recent findings in embryonic amniotes, we hypothesize that preferential pH_i regulation may represent a general pattern of acid–base regulation in vertebrate embryos that is either retained or lost during development, likely depending upon acid–base challenges that these groups have experienced during adaptation to their environment. Consequently, we propose that many other vertebrates, including those that exhibit coupled pH regulation as adults, may rely exclusively on preferential pH_i regulation during development; an area clearly worthy of further investigation. Finally, we hypothesize that the retention of preferential pH_i regulation may have been an exaptation for a number of important evolutionary transitions in vertebrates, including the invasion of freshwater, the evolution of air breathing and the transition to land, events where acid–base homeostasis would otherwise be impaired (Brauner and Baker,

Table 2. Pattern of vertebrate pH regulation following exposure to an acute (<48 h) respiratory acidosis of ≥ 1 kPa blood P_{CO_2}

Group	Species	Pattern of pH regulation	Comments	References
Cyclostomata	Pacific hagfish (<i>Eptatretus stoutii</i>)	Coupled pH		Baker et al., 2015
Chondrichthyes	Atlantic big skate (<i>Raja ocellata</i>)	Coupled pH		Wood et al., 1990
Dipnoi	South American lungfish (<i>Lepidosiren paradoxa</i>)	Preferential pH_i	Indirect determination based on CO_2 tolerance and pH_e measurement	Sanchez et al., 2005; Shartau and Brauner, 2014
Amphibia	Cane toad (<i>Bufo marinus</i>) Greater siren (<i>Siren lacertina</i>)	Coupled pH Preferential pH_i		Snyder and Nestler, 1991 Heisler et al., 1982
Mammalia	Rat (<i>Rattus norvegicus</i>) Hamster (<i>Cricetus cricetus</i>) Guinea pig (<i>Cavia porcellus</i>) Dog (<i>Canis lupus familiaris</i>) Dog (<i>Canis lupus familiaris</i>) Cat (<i>Felis catus</i>)	Coupled pH Coupled pH Coupled pH Coupled pH Coupled pH Coupled pH	Newborn	Gonzalez and Clancy, 1986; Litt et al., 1985 Malan et al., 1985 Wood and Schaefer, 1978 Nattie and Edwards, 1981 Arieff et al., 1976 Yaksh and Anderson, 1987
Testudines	Common snapping turtle (<i>Chelydra serpentina</i>) Western painted turtle (<i>Chrysemys picta bellii</i>)	Preferential pH_i Coupled pH	Embryo (70 and 90% to hatch) and yearling	Shartau et al., 2016 Wasser et al., 1991
Archosauria	American alligator (<i>Alligator mississippiensis</i>)	Preferential pH_i	Embryo (70% to hatch)	R.B.S., D.A.C., Z. F. Kohl, R. M. Elsey and C.J.B., unpublished
Lepidosauria	Knight anole (<i>Anolis equestris</i>) Desert iguana (<i>Dipsosaurus dorsalis</i>)	Coupled pH Coupled pH		Snyder et al., 1995 Snyder et al., 1995
Acipenseriformes	White sturgeon (<i>Acipenser transmontanus</i>)	Preferential pH_i		Baker et al., 2009
Lepisosteiformes	Spotted gar (<i>Lepisosteus oculatus</i>) Longnose gar (<i>L. osseus</i>) Alligator gar (<i>Atractosteus spatula</i>)	Preferential pH_i Preferential pH_i Preferential pH_i		Shartau and Brauner, 2014 Shartau and Brauner, 2014 Shartau and Brauner, 2014
Amiiformes	Bowfin (<i>Amia calva</i>)	Preferential pH_i	Indirect determination based on CO_2 tolerance and pH_e measurement	Brauner and Baker, 2009
Otomorpha	Armoured catfish (<i>Pterygoplichthys pardalis</i>) Striped catfish (<i>Pangasianodon hypophthalmus</i>) Channel catfish (<i>Ictalurus punctatus</i>)	Preferential pH_i Preferential pH_i Coupled pH	Hypercarbia in low pH water (4.5 pH units)	Brauner et al., 2004 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 Cameron, 1980
Euteleosteomorpha	Marbled swamp eel (<i>Synbranchus marmoratus</i>) Lemon sole (<i>Parophrys vetulus</i>) Cod (<i>Gadus morhua</i>) Trout (<i>Oncorhynchus mykiss</i>)	Preferential pH_i Coupled pH Coupled pH Coupled pH		Heisler, 1982 Wright et al., 1988 Larsen et al., 1997 Wood and LeMoigne, 1991

Studies used adult animals unless indicated otherwise.

2009; Janis et al., 2012; Shartau and Brauner, 2014). The specific cellular and molecular mechanisms of preferential pH_i regulation are currently unknown and represent a fascinating opportunity for further investigation. The role of preferential pH_i regulation during

development and vertebrate evolution are areas for research that remain largely unexplored, and experiments addressing these issues will likely provide valuable insight into what may represent a basal pattern of acid–base regulation in vertebrates.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.B.S. wrote the manuscript. All authors discussed and edited the manuscript.

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