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RESEARCH ARTICLE

Embryonic common snapping turtles (*Chelydra serpentina*) preferentially regulate intracellular tissue pH during acid–base challenges

Ryan B. Shartau[1,*], Dane A. Crossley, II[2], Zachary F. Kohl[2] and Colin J. Brauner[1]

ABSTRACT

The nests of embryonic turtles naturally experience elevated CO_2 (hypercarbia), which leads to increased blood P_{CO_2} and a respiratory acidosis, resulting in reduced blood pH [extracellular pH (pH_e)]. Some fishes preferentially regulate tissue pH [intracellular pH (pH_i)] against changes in pH_e; this has been proposed to be associated with exceptional CO_2 tolerance and has never been identified in amniotes. As embryonic turtles may be CO_2 tolerant based on nesting strategy, we hypothesized that they preferentially regulate pH_i, conferring tolerance to severe acute acid–base challenges. This hypothesis was tested by investigating pH regulation in common snapping turtles (*Chelydra serpentina*) reared in normoxia then exposed to hypercarbia (13 kPa P_{CO_2}) for 1 h at three developmental ages: 70% and 90% of incubation, and yearlings. Hypercarbia reduced pH_e but not pH_i, at all developmental ages. At 70% of incubation, pH_e was depressed by 0.324 pH units while pH_i of brain, white muscle and lung increased; heart, liver and kidney pH_i remained unchanged. At 90% of incubation, pH_e was depressed by 0.352 pH units but heart pH_i increased with no change in pH_i of other tissues. Yearlings exhibited a pH_e reduction of 0.235 pH units but had no changes in pH_i of any tissues. The results indicate common snapping turtles preferentially regulate pH_i during development, but the degree of response is reduced throughout development. This is the first time preferential pH_i regulation has been identified in an amniote. These findings may provide insight into the evolution of acid–base homeostasis during development of amniotes, and vertebrates in general.

KEY WORDS: Acid–base regulation, Development, Reptile, Preferential pH_i regulation, Hypercarbia, Amniotes, Physiology, CO_2

INTRODUCTION

The nests of many reptiles naturally experience changes in CO_2 levels, often resulting in an elevated CO_2 (hypercarbia) rearing environment for the embryos. These conditions arise because of a number of biotic and abiotic factors, including nest saturation from precipitation, metabolic activity of microorganisms and 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 P_{CO_2} (partial pressure of CO_2) (Booth, 1998; Prange and Ackerman, 1974); similar P_{CO_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 hypercarbia-induced respiratory acidosis has been well described in adult amniotes, and it is typically characterized by an initial reduction in both blood pH [extracellular pH (pH_e)] and tissue pH [intracellular pH (pH_i)], which change in a qualitatively similar manner. The recovery of pH_i usually precedes that of pH_e, but recovery in the two 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 recovery 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 acid–base disturbances (Brauner and Baker, 2009; Shartau and Brauner, 2014).

Chicken embryos between 60% and 90% of incubation subjected to hypercarbia (5 kPa P_{CO_2}) for 24 h experienced a reduction in pH_e that was largely uncompensated (Burggren et al., 2012). Embryonic chickens are exceptionally hypercarbia tolerant as they can survive 1 h exposure to P_{CO_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, which has not been previously investigated 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 probably 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, series 1 experiments investigated the pattern of acid–base regulation in normocarbia/normoxia-reared animals

[1]Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4. [2]Department of Biological Sciences, University of North Texas, Denton, TX 76203-5017, USA.

*Author for correspondence (shartau@zoology.ubc.ca)

R.B.S., 0000-0002-0455-8496

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List of symbols and abbreviations

HC13	hypercarbic hypoxia (13 kPa P_{CO_2}, 9 kPa P_{O_2})
HC3.5	hypercarbia (3.5 kPa P_{CO_2}, 21 kPa P_{O_2}) condition
NC	normocarbia (0.03 kPa P_{CO_2}, 21 kPa P_{O_2}) condition
P_{CO_2}	partial pressure of CO_2
pH_e	blood (extracellular) pH
pH_i	tissue (intracellular) pH
P_{O_2}	partial pressure of O_2

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 in whether 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.

MATERIALS AND METHODS

Turtle embryo acquisition and incubation

Common snapping turtle [*Chelydra serpentina* (Linnaeus 1758)] eggs were collected in north-western Minnesota, USA, and transported by automobile to the lab at the University of North Texas (Minnesota Department of Natural Resources Permit No. 19772 to D.A.C.). Eggs were staged to determine the approximate age of each clutch (53–55 days total incubation period at 30°C; Yntema, 1968). Eggs were incubated at 30°C in a walk-in, constant-temperature room on a 14 h:10 h light:dark photoperiod. All embryos were incubated in plastic containers placed on 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 P_{CO_2}, 21 kPa P_{O_2}; NC) or hypercarbic/normoxic (3.5 kPa P_{CO_2}, 21 kPa P_{O_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 Ziploc 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, USA). HC3.5 embryos were incubated in separate 0.3 m^3 Percival incubators (model I30NLX, Percival Scientific) 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 P_{CO_2}, 21 kPa P_{O_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 analysers (S-A/I and CD-3A, respectively; Ametek Applied Electrochemistry, IL, USA) connected to a PowerLab® with LabChart Pro® software (v7, 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 1 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 the University of North Texas IACUC no. 11-007.

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 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, 2000). The surgical preparations were minimally invasive and no anaesthesia/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°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 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 was acquired at 40 Hz using PowerLab data recording system (ADInstruments, CO, USA) connected to a computer running Chartpro software (v7.4, 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°C by

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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.

Experimental treatments

Series 1: acid-base status in normocarbia/normoxia-reared animals exposed 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 detailed 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 of severe hypercarbic hypoxia (13 kPa P_{CO_2} and 9 kPa P_{O_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 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 a command module (model SDPROC, Aalborg) 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 analysers (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: acid–base regulation at 90% of incubation in embryos reared under constant hypercarbia and exposed to respiratory acidosis or alkalosis

HC3.5-reared embryos at 90% of incubation were sampled directly from HC3.5 to examine the effect of hypercarbic rearing on acid–base balance at CO_2 tensions likely to be 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 sampling 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.

Because of 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.

Blood sampling, 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 1 ml heparinized plastic syringe; blood pH (pH_e) and total CO_2 (T_{CO_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). T_{CO_2} was measured using a total CO_2 analyser (Corning model 965 Analyzer, Essex, UK) and was calibrated using freshly prepared 0, 10 and 25 mmol l^{-1} $NaHCO_3$. Embryos were then killed with an overdose of sodium pentobarbital (100 mg kg^{-1}) injected into the CAM artery. Tissues (heart, brain, liver, white muscle, kidney and lung) were 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 subsequently ground under liquid nitrogen and pH_i was measured using the metabolic inhibitor tissue homogenate 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 Stat Profile Prime (Nova Biomedical, Waltham, MA, USA).

Yearlings

To sample blood and tissues in yearlings, turtles were removed from the chamber, killed with an overdose of isoflurane and the plastron removed and the heart exposed. Blood was sampled (~200–300 µl) using a 30 gauge heparinized 1 ml syringe from the right aorta. 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. Because of the greater blood volume collected in yearlings, blood P_{CO_2} was measured at the same time as pH_e using a P_{CO_2} electrode (E201/E5037; Loligo Systems, Denmark) thermostated at 30°C in a Radiometer BMS 3 MK 2 calibrated daily with humidified pre-mixed gases. All measurements of pH_i, pH_e and T_{CO_2} were taken as described above.

Calculations and statistical analyses

Plasma [HCO_3^-] and P_{CO_2} were calculated using measured T_{CO_2} and pH values as described by Brauner et al. (2004). The CO_2 solubility coefficient and the logarithmic acid dissociation constant (pK_a) 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 is plotted as mean values±s.e.m.

All data were analysed 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–Wilk 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 a 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 analysed using the Kruskal–Wallis test (P<0.05). Absolute blood pressure was corrected for the distance of the pressure transducer 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

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compared with unexposed measurements using a one-way ANOVA, followed by a Tukey *post hoc* (P<0.05). All values are presented as means±s.e.m.; sample size was N=8 for NC embryos, N=6 for NC yearlings and N=6 for HC3.5 embryos. All figures were created using GraphPad Prism v5.0 (GraphPad Software Inc. 2007).

RESULTS

Acid–base regulation in embryos

Series 1: acid–base status in normocarbia/normoxia-reared animals exposed to severe hypercarbic hypoxia

NC-reared animals transferred to HC13 for 1 h exhibited a significant reduction in pH_e and a significant increase in blood P_{CO_2} at all three developmental ages (Welch 2-sample *t*-test, P<0.05; Fig. 1A) as expected *a priori*. Blood [HCO_3^-] did not change significantly (Fig. 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 the heart, liver or kidney (Fig. 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. 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. 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 at each developmental age. There was a statistically significant effect of developmental age on the difference in tissue [H^+] from control,

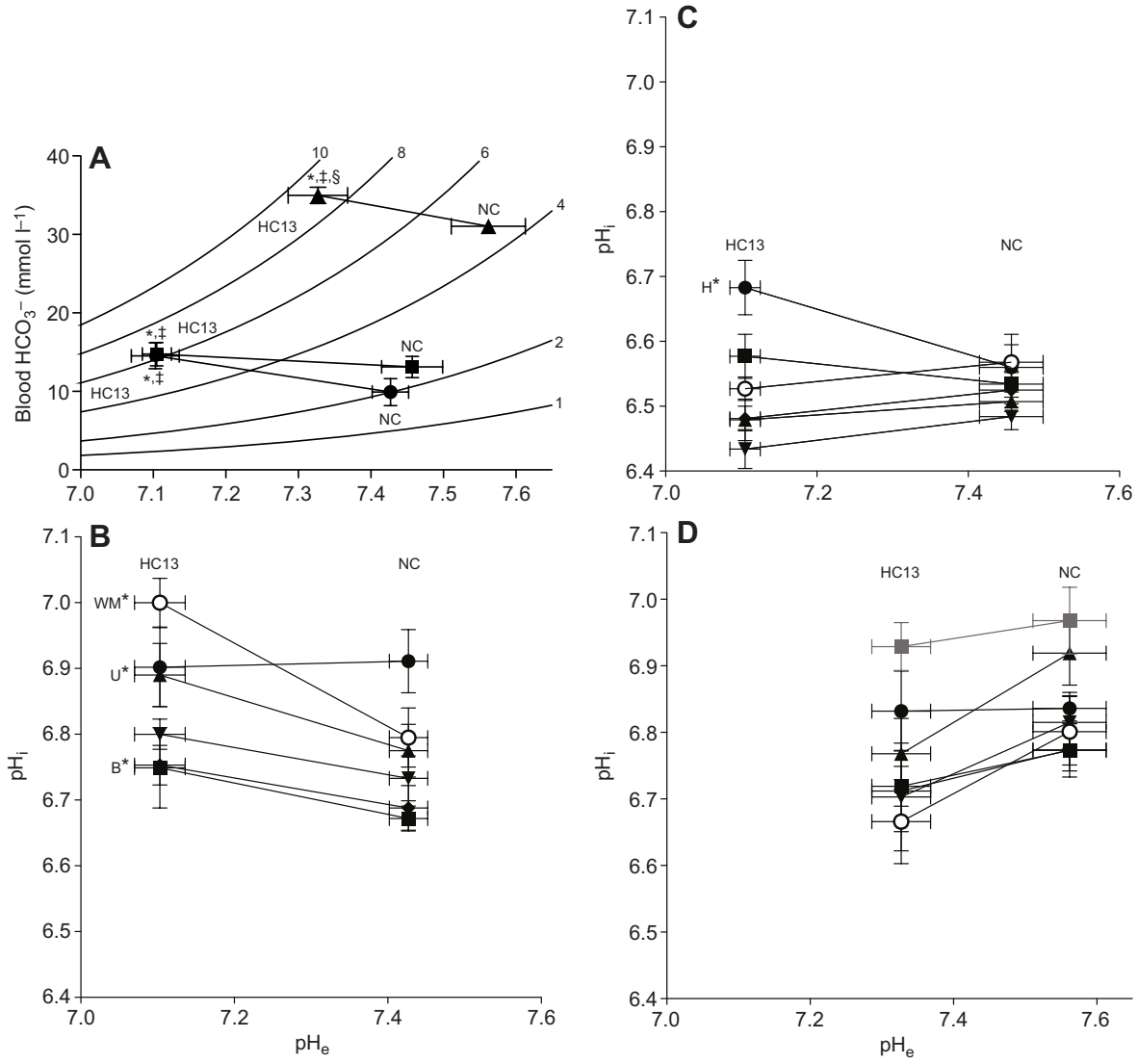

Fig. 1. Effect of exposure of common snapping turtle (*Chelydra serpentina*) embryos and yearlings to an acute respiratory acidosis on blood and tissue acid–base status (series 1). (A) Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH–HCO_3^- plot. Embryos at 70% (●) or 90% of incubation (■), or yearlings (▲) were sampled in normocarbia (0.03 kPa P_{CO_2}, 21 kPa P_{O_2}; NC) or following 1 h hypercarbic hypoxia (13 kPa P_{CO_2}, 9 kPa P_{O_2}; HC13) exposure; curved lines represent P_{CO_2} isopleths (kPa). (B–D) The relationship between pH_e and tissue pH (pH_i) in snapping turtles at 70% of incubation (B), 90% of incubation (C), and in yearlings (D) following 1 h exposure to HC13. Tissues are indicated by the following symbols: heart, ●; liver, ■; lung, ▲; kidney, ▼; brain, ◆; white muscle, ○; and red blood cell, ■ (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 P_{CO_2} and §blood HCO_3^-. B–D: *significant differences in pH_i from the NC group for the indicated tissue (H, heart; WM, white muscle; U, lung; B, brain) (P<0.05).

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where a progressive 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 tissues differ significantly when developmental age is considered; two-way ANOVA, $P<0.05$; Fig. 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 from 53.2±4.6 beats min^{-1} to 36.7±2.7 beats min^{-1}, respectively (one-way ANOVA, Tukey's *post hoc*, $P<0.001$).

Series 2: acid–base regulation at 90% of incubation in embryos reared under constant hypercarbia and exposed to respiratory acidosis or alkalosis

Embryos at 90% of incubation reared at HC3.5 had increased pH_e, blood P_{CO_2} and [HCO_3^-] compared with those reared in NC (Fig. 3A–C). pH_i was also significantly elevated in all tissues, except liver (Fig. 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 P_{CO_2} but no change in blood [HCO_3^-] (Welch 2-sample *t*-test, $P<0.001$; Fig. 4A). Heart pH_i was significantly reduced; there was no change in the other tissues (Welch 2-sample *t*-test, $P<0.05$; Fig. 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

Table 1. Plasma ion concentration at 90% of incubation in 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 P_{CO_2}, 21 kPa P_{O_2}; $N=8$); HC3.5, hypercarbia (3.5 kPa P_{CO_2}, 21 kPa P_{O_2}; $N=6$).
Data are means±s.e.m. *Significant difference between rearing conditions ($P<0.05$).

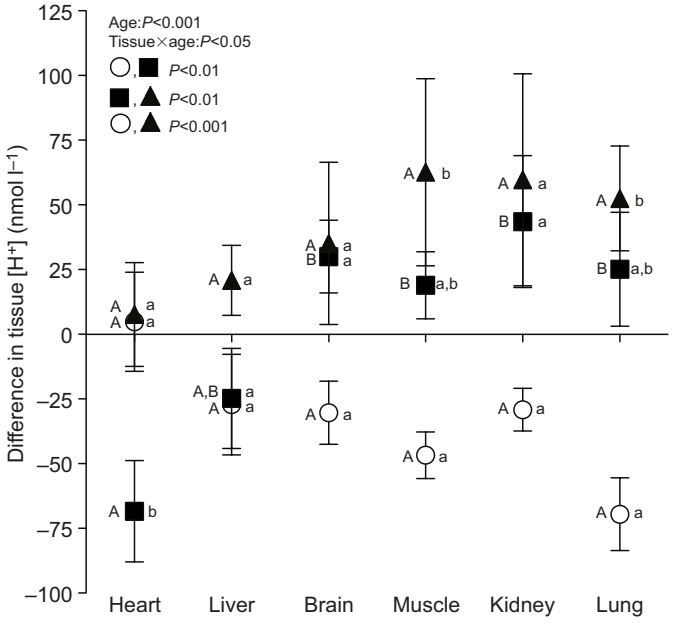

Fig. 2. Difference in tissue [H^+] from control following 1 h exposure of common snapping turtle to hypercarbic hypoxia (series 1). Hypercarbic hypoxia: 13 kPa P_{CO_2}, 9 kPa P_{O_2} (HC13); normocarbia control: 0.03 kPa P_{CO_2}, 21 kPa P_{O_2} (NC). Concentrations of H^+ were calculated from tissue pH ([H^+]=10^{-pH}) and the mean NC [H^+] was subtracted from individual HC13 [H^+] values to calculate the mean [H^+] difference ±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 two-way ANOVA, followed by Tukey's *post hoc* ($N=8$ for 70% and 90% of incubation, and $N=6$ for yearlings). Different uppercase letters indicate significant differences between tissues for the same developmental age; different lowercase letters indicate significant differences between developmental age in the same tissue following separate one-way ANOVA followed by Tukey's *post hoc* ($P<0.05$).

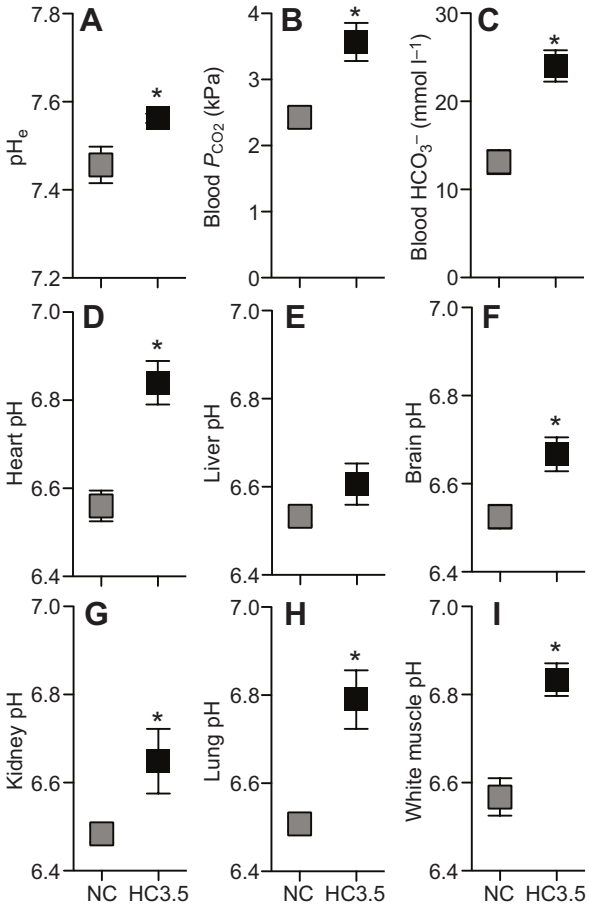

Fig. 3. Changes in blood and tissue acid–base status in common snapping turtle embryos at 90% of incubation continuously reared in either normocarbia or hypercarbia (series 2). (A) Blood pH (pH_e), (B) blood P_{CO_2}, (C) blood HCO_3^-, (D) heart pH, (E) liver pH, (F) brain pH, (G) kidney pH, (H) lung pH and (I) white muscle pH. Black squares, normocarbia (0.03 kPa P_{CO_2}, 21 kPa P_{O_2}; NC); grey squares, hypercarbia (3.5 kPa P_{CO_2}, 21 kPa P_{O_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 Figs 1 and 4. *Significant differences between rearing conditions ($P<0.05$).

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acid–base status between the groups. The HC3.5-reared embryos had a greater [K^+] compared with the NC-reared embryos (*t*-test, $P<0.05$). There were no differences in the other ion concentrations (Table 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 a reduction in blood P_{CO_2} (one-way ANOVA, Tukey's *post hoc*, $P<0.001$; Fig. 5A). There was a significant reduction in [HCO_3^-] following 24 h NC exposure (one-way ANVOA, Tukey's *post hoc*, $P<0.01$; Fig. 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. 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 from 58.1±1.3 beats min^{-1} to 39.6±1.5 beats min^{-1}, respectively (one-way ANOVA, Tukey's *post hoc*, $P<0.001$).

DISCUSSION

Preferential pH_i regulation has been documented in a number of fishes, and in an aquatic salamander, but never before in amniotes (Cameron, 1989; Everaert et al., 2011; Shartau and Brauner, 2014).

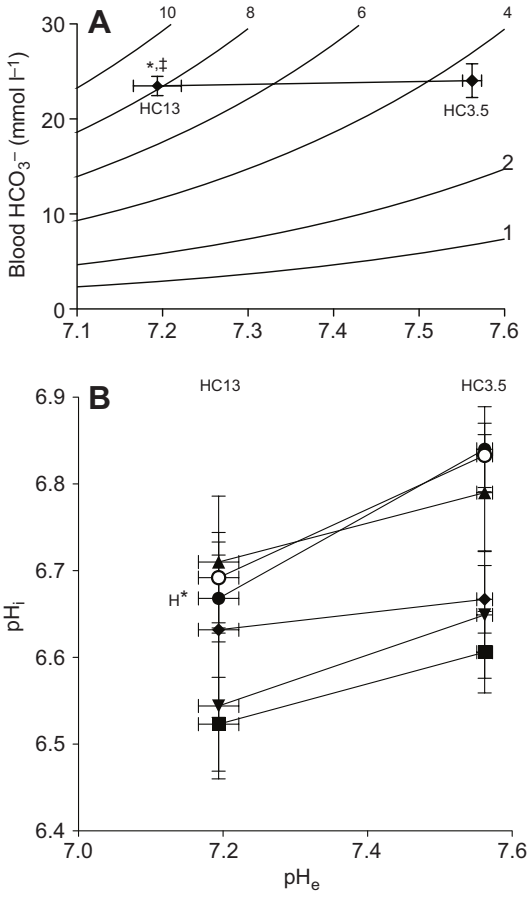

Fig. 4. Effect of exposure to hypercarbic hypoxia in snapping turtle embryos at 90% of incubation reared continuously in hypercarbia (series 2). Hypercarbia: 3.5 kPa P_{CO_2}, 21 kPa P_{O_2} (HC3.5); hypercarbic hypoxia: 13 kPa P_{CO_2}, 9 kPa P_{O_2} (HC13). (A) Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH–HCO_3^- plot. Embryos were sampled in HC3.5 or following 1 h HC13 exposure; curved lines represent P_{CO_2} isopleths (kPa). (B) The relationship between pH_e and tissue pH (pH_i) in snapping turtles following 1 h exposure to HC13. Tissues are indicated by the following symbols: heart, ●; liver, ■; lung, ▲; kidney, ▼; brain, ◆; and white muscle, ○. 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 P_{CO_2}. B: *significant differences in pH_i from the HC3.5 group for the indicated tissue (H, heart) ($P<0.05$).

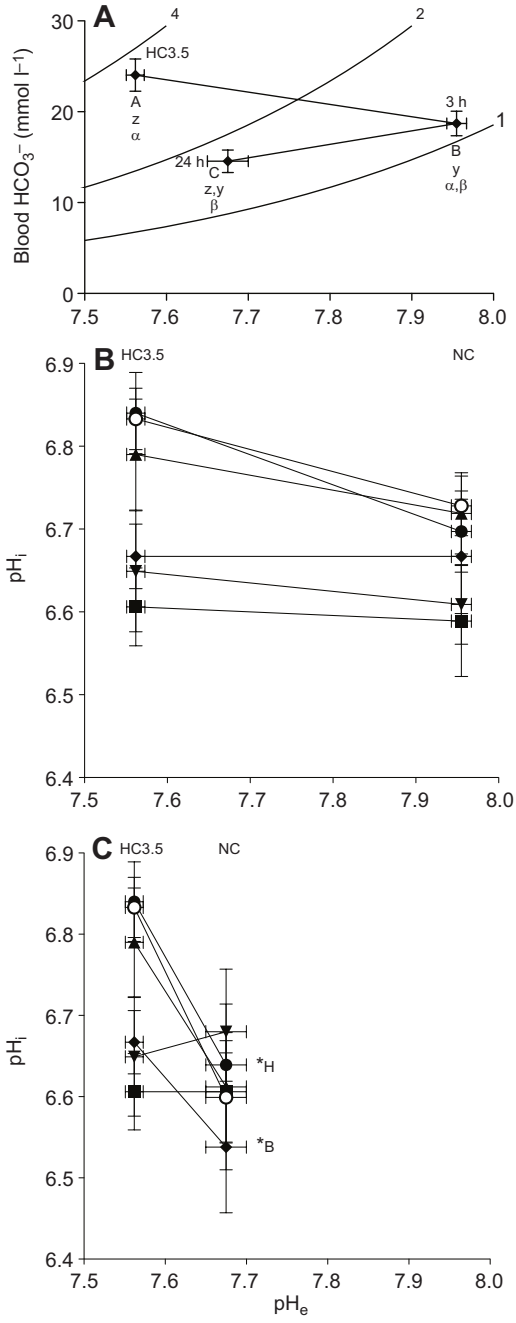

Fig. 5. Effect of exposure to normocarbia in snapping turtle embryos at 90% of incubation reared continuously in hypercarbia (series 2). Hypercarbia: 3.5 kPa P_{CO_2}, 21 kPa P_{O_2} (HC3.5); normocarbia: 0.03 kPa P_{CO_2}, 21 kPa P_{O_2} (NC). (A) Blood pH (pH_e) and blood [HCO_3^-] are presented on a pH–HCO_3^- plot. Embryos were sampled in HC3.5 or following a 3 h or 24 h NC exposure; curved lines represent P_{CO_2} isopleths (kPa). (B,C) The relationship between pH_e and tissue pH (pH_i) in snapping turtles following exposure to NC for 3 h (B) or 24 h (C). Tissues are indicated by the following symbols: heart, ●; liver, ■; lung, ▲; kidney, ▼; brain, ◆; and white muscle, ○. A: different letters indicate significant differences ($P<0.05$) between control (HC3.5) and treatment (NC) for pH_e (uppercase letters), blood P_{CO_2} (lowercase letters) and blood HCO_3^- (Greek letters). C: *significant difference in pH_i from the HC3.5 group for the indicated tissue (H, heart; B, brain) ($P<0.05$).

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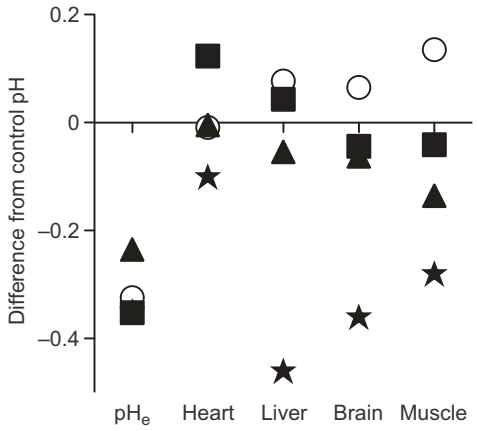

Fig. 6. Difference in blood and tissue pH of turtles during development following exposure to hypercarbia relative to normocarbia in common snapping turtles and western painted turtles. Data for common snapping turtles at 70% and 90% of incubation and yearlings are from this study; data for adult western painted turtles, *Chrysemys picta bellii*, are from 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 P_{CO_2}, 9 kPa P_{O_2}) exposure (from Fig. 1) in snapping turtles or 1 h 6.5 kPa P_{CO_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 with either embryos or yearlings in the present study. Symbols: 70% of incubation, ○; 90% of incubation, ■; yearlings, ▲; and adult western painted turtle, ★.

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 probably important for tolerating acute respiratory acid–base disturbances in this amniote species.

Capacity for preferential pH_i regulation shifts during development

Exposure of NC-reared turtles to HC13 greatly increased blood P_{CO_2} (Fig. 1A); the difference between blood and environmental P_{CO_2} of 13 kPa probably represents non-equilibrium between the animals and the environment due to the short exposure time. Despite the lack of complete CO_2 equilibration, turtles experienced large reductions in pH_e (which was the objective of the treatment) but there was no reduction in pH_i (Fig. 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 embryos at 90% of incubation and not at all in yearlings (Fig. 1B–D), suggesting younger embryos possess a greater capacity for preferential pH_i regulation. When contrasted with findings in 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 hypercapnia-exposed and control animals is plotted for blood and tissues (Wasser et al., 1991) in Fig. 6, along with relevant results from this study to highlight the large pH_i reductions in adult turtles compared with embryos (Fig. 6).

The differences in the pattern of acid–base regulation between snapping turtle embryos and yearlings, and western painted turtle adults is probably due to changes in the capacity for preferential pH_i regulation and buffering capacity. An increase in pH_i from control values during acidosis (or a decrease during 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 acidosis (or increases during alkalosis). Turtles appear to transition from preferentially regulating pH_i to having coupled pH regulation.

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 P_{CO_2} of 3.6 kPa P_{CO_2} (Fig. 3B), which was slightly higher than the incubation P_{CO_2} of 3.5 kPa P_{CO_2}. This indicates that these embryos were in equilibrium with environmental P_{CO_2}, as would be expected, and the slightly higher blood P_{CO_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 with NC-reared embryos (Fig. 3A,C), suggesting these embryos have compensated pH_e in chronic hypercarbia; pH_i was also elevated in all tissues, except liver (Fig. 3D–I). The increase in blood HCO_3^- (Fig. 3C) and plasma K^+ (Table 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.

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 (2005) found that early stage embryonic cells of zebrafish (*Danio rerio*) regulate pH_i when exposed to changes in ambient P_{CO_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, we investigated the response of American alligator embryos to severe respiratory acidosis and found that they also preferentially regulate pH_i, similar to the turtle embryos shown here (R.B.S., D.A.C., Z.F.K., R. M. Elsey and C.J.B., unpublished).

During ontogeny, the capacity for coupled pH regulation increases as a result of 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 unit.

Snapping turtle embryos and yearlings are tolerant to 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 P_{CO_2} (Baker et al., 2009a; Brauner and Baker, 2009; Shartau and Brauner, 2014; this study). 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 because of 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 P_{CO_2} (Brauner and Baker, 2009). In addition to conferring exceptional tolerance to hypercarbia-induced acidosis, preferential pH_i regulation appears to play a role in short-term pH_i regulation during metabolic acidosis, metabolic alkalosis (Harter et al., 2014) and respiratory alkalosis (Fig. 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 resulting from HC13 exposure (Fig. 1). Additionally, embryos reared at 3.5 kPa P_{CO_2}, which probably mirrors natural nest conditions, largely maintained pH_i during both HC13 and NC exposure, which created an acidosis and alkalosis, respectively (Figs 4 and 5); this suggests that preferential pH_i regulation is a pattern of acid–base regulation that is used during the course of development, conferring a 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. The difference in cardiac function between developmental 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 periods of lower metabolic demand (Jackson, 1987; Jackson et al., 1991).

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 demonstrate the first occurrence of preferential pH_i regulation in an amniote; furthermore, we also found the capacity for preferential pH_i regulation changes during development from embryo to yearling. Preferential pH_i regulation in developing snapping turtles and other amniotes, such as American alligators (R.B.S., D.A.C., Z.F.K., R. M. Elsey and C.J.B., unpublished), probably 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 whether 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.B.S., D.A.C. and C.J.B. designed the study. R.B.S., D.A.C. and Z.F.K. executed the experiments. R.B.S. wrote the manuscript. All authors discussed the results and revised the manuscript.

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Data availability

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