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American alligator (*Alligator mississippiensis*) embryos tightly regulate intracellular pH during a severe acidosis

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Abstract

Crocodylian nests naturally experience high CO₂ (hypercarbia), which leads to increased blood PCO₂ and reduced blood pH (pH_e) in embryos; their response to acid-base challenges is not known. During acute hypercarbia, snapping turtle embryos preferentially regulate tissue pH (pH_i) against pH_e reductions. This is proposed to be associated with CO₂ tolerance in reptilian embryos and is not found in adults. In the present study, we investigated pH regulation in American alligator *Alligator mississippiensis* (Daudin, 1802) embryos exposed to 1 h hypercarbia hypoxia (13 kPa PCO₂, 9 kPa PO₂). Hypercarbia hypoxia reduced pH_e by 0.42 pH units while heart and brain pH_i increased, with no change in pH_i of other tissues. The results indicate American alligator embryos preferentially regulate pH_i, similar to snapping turtle embryos, which represents a markedly different strategy of acid-base regulation than what is observed in adult reptiles. These findings suggest that preferential pH_i regulation may be a strategy of acid-base regulation used by embryonic reptiles.

Les nids de crocodilien connaissent naturellement un CO₂ élevé (hypercarbie), ce qui entraîne une augmentation du PCO₂ dans le sang et un pH sanguin (pH_e) réduit chez les embryons; leur réponse aux défis de acide-base n'est pas connue. Au cours de l'hypercarbie aiguë, les embryons de tortues isolées régulent de manière préférentielle le pH des tissus (pH_i) contre les réductions de pH_e. Ceci est proposé pour être associé à la tolérance au CO₂ dans les embryons reptiliens et ne se trouve pas chez les adultes. Dans la présente étude, nous avons étudié la régulation du pH chez les embryons de l'alligator

américain *Alligator mississippiensis* (Daudin, 1802) exposés à une hypoxie hypercarbie 1
h (13 kPa PCO_2 , 9 kPa PO_2). L'hypercarbie hypoxie a réduit le pH_e par 0,42 unité de pH,
tandis que le pH_i du cœur et du cerveau a augmenté, sans changement de pH d'autres
tissus. Les résultats indiquent que les embryons de l'alligator américain régulent
préférentiellement le pH_i , semblable à les embryons de tortues, ce qui représente une
stratégie nettement différente de régulation acide-base que ce qui est observé chez les
reptiles adultes. Ces résultats suggèrent que la régulation préférentielle du pH_i peut être
une stratégie de régulation acide-base utilisée par les reptiles embryonnaires.

Keywords: *Alligator mississippiensis*; American alligator; acid-base physiology;
development; reptile; preferential pH_i regulation; hypercarbia; amniotes

Introduction

Acid-base regulation in adult amniotes relies on net H^+ exchange with the environment through ventilatory and/or renal pathways (Cameron 1989), however, during embryonic development of oviparous animals this is constrained as the egg shell structure limits environmental interaction (Erasmus et al. 1971; Everaert et al. 2011; Eme and Crossley 2015). Acid-base balance is one of the most important physiological variables and tight pH regulation is critical as small deviations can have large effects on molecular function, and ultimately reduce whole animal performance (Cameron 1989; Putnam and Roos 1997; Shartau et al. 2016a). In adult amniotes, the recovery of intracellular pH (pH_i) following an acid-base disturbance may precede that of blood pH (extracellular pH; pH_e) but complete pH_i recovery is dependent upon significant (>50%) pH_e recovery. Thus, pH_i recovery is coupled to pH_e recovery, which has been termed coupled pH regulation (Shartau et al. 2016a).

Acid-base regulation in adult amniotes is characterized by the strategy of coupled pH regulation (Shartau et al. 2016a). However, the response in embryonic amniotes, which are constrained within an egg shell, is poorly understood (Everaert et al. 2011; Eme and Crossley 2015). Limited evidence from snapping turtle embryos *Chelydra serpentina* (L., 1758) 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 , but remarkably, was associated with no change in pH_i indicating the latter was completely protected (Shartau et al. 2016b). The ability to completely regulate pH_i in the face of a pronounced and

maintained reduction in pH_e in other vertebrates is referred to as preferential pH_i regulation and is defined as a $\Delta\text{pH}_i/\Delta\text{pH}_e \leq 0$ during an acidosis (Heisler 1984; Brauner and Baker 2009). Preferential pH_i regulation has only previously been observed in a few adult anamniotes (e.g. white sturgeon [*Acipenser transmontanus* Richardson 1836], armoured catfish [*Pterygoplichthys pardalis* (Castelnau 1855)] and greater siren [*Siren lacertina* L., 1767]) but never in adult amniotes (Erasmus et al. 1971; Everaert et al. 2011; Shartau and Brauner 2014; Eme and Crossley 2015; Shartau et al. 2016b).

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 (Everaert et al. 2011; Eme and Crossley 2015; Shartau et al. 2016b). Consequently, preferential pH_i regulation may be a trait of embryos and has been observed in the snapping turtle, *C. serpentina*, embryos. We propose that this may be especially the case in embryos 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 (Lutz and Dunbar-Cooper 1984; Grigg et al. 2010); 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 (Lutz and Dunbar-Cooper 1984; Grigg et al. 2010). 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 CO₂ 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*. Therefore, *Alligator mississippiensis* (Daudin, 1802) embryos were exposed to hypercarbic/hypoxic conditions to induce a severe acute respiratory metabolic acidosis. The impact of this exposure on blood and tissue acid-base status was examined to gain insight into the pattern of pH regulation in embryonic crocodilians and to determine whether they also employ preferential pH_i regulation.

Material and methods

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 where they were incubated at 30°C in a walk-in incubator ensuring all embryos developed as female. The developmental stage of a clutch upon arrival was determined by a combination of candling and dissections and then estimates from that point onwards were calculated based upon accumulated thermal units (days of incubation × temperature of incubation). 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.

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°C) under normoxic/normocarbic conditions and ~1 cm² 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 was 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 cm³ per chamber, one embryo per chamber) and allowed to acclimate for at least 60 minutes. Temperature in the chambers was maintained at 30°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 (ADInstruments model MLT0699, CO, USA) 1-3 cm above the egg via saline-filled PE50 tubing, connected to an amplifier (Quad Bridge Amp, ADInstruments, CO, USA), 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.

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 time to hatch (referred to as 70% of incubation from this point forward) at which time the mean embryo weight was 15.7 ± 0.5 g. The embryos within the egg were then subjected to exposed for 1 h to either air or hypercarbic hypoxia (13 kPa PCO_2 and 9 kPa PO_2 ; H13). A 1 h exposure duration to hypercarbia was chosen to depress pH_e and elevate tissue CO_2 to look for preferential pH_i regulation as it is known that the egg shell is very gas permeable (Tazawa et al. 1988) and in chicken embryos impairment of CO_2 excretion through submergence of the egg in water resulted in a rapid pH_e depression of 0.5 pH units within 20 min (Andrewartha et al. 2014). Exposure to 13% CO_2 , as done here, would likely rapidly increase internal CO_2 through diffusion from the environment, while simultaneously impairing CO_2 elimination until the embryo reached a new steady state. Based upon the findings of Andrewartha et al. (2014), and assuming alligator embryos would behave similar to that of chicken, a pronounced elevation in CO_2 would be achieved < 20 min allowing time for blood and tissue CO_2 levels to equilibrate. H13 was 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, approximately 150-300 μL of blood was sampled from the CAM artery by disconnecting the pressure catheter to allow blood to passively flow into a 1 mL heparinized syringe. Blood was then separated into two aliquots with the first aliquot being used to immediately measure blood pH (pH_e ; model BMS 3 MK 2; Radiometer; Copenhagen, Denmark). The second aliquot was centrifuged (3 min at 10,000 rpm) and plasma was removed for immediate measurement of total CO_2 (TCO_2) (Corning 965 TCO_2 Analyzer; Essex, UK) 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. This technique has been validated (Portner et al. 1990; Baker et al. 2009) and has been previously used in reptiles (Galli and Richards 2012; Shartau et al. 2016b). RBC pH_i was measured using the freeze-thaw technique (Baker et al. 2009).

Calculations and statistical analyses

Plasma $[\text{HCO}_3^-]$ 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 pK_a 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 $\pm$ s.e.m.

All data were analyzed using GraphPad Prism v5.0 (GraphPad Software Inc., 2007). Differences between the acid-base variables 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 $\pm$ s.e.m.; sample sizes are $n=6-7$ except for RBC where $n=5$.

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 ($t_{(10)}=5.02$, $P < 0.001$), which was accompanied by reductions in pH_e ($t_{(13)}=12.25$, $P < 0.001$) and RBC ($t_{(8)}=3.17$, $P=0.013$); blood $[HCO_3^-]$ did not differ (Fig. 1A) ($t_{(13)}=0.455$, $P=0.657$). Despite pH_e being reduced by 0.506 pH units, pH_i of tissues was not reduced; heart ($t_{(10)}=2.71$, $P=0.022$) and brain ($t_{(10)}=2.38$, $P=0.039$) pH_i increased (0.226 and 0.181 pH units, respectively) (Fig. 1B), while no change in pH_i of liver ($t_{(10)}=0.13$, $P=0.895$), white

muscle ($t_{(10)}=1.43$, $P=0.179$), or kidney ($t_{(10)}=0.479$, $P=0.642$) were observed (Fig. 1C).

Interestingly, these pH_i values are relatively low (Fig. 1B,C) compared to adult vertebrates (Shartau et al. 2016a) but are comparable to embryonic turtles (Shartau et al. 2016b), the only other study to measure pH_e and pH_i in an embryonic amniote.

The statistical conclusions were the same whether the analyses were conducted on pH values or converted $[\text{H}^+]$ (Fig. 1D,E), consistent with the findings of Boutilier and Shelton (1980) who conclude that the use of pH is as valid as that of $[\text{H}^+]$ for statistical analysis. They suggest this conclusion is applicable to all vertebrates that have fairly precise pH (or $[\text{H}^+]$) regulation, which appears to be the case here.

Exposure to elevated CO_2 was expected to reduce pH_e , however, a pure respiratory acidosis would be associated with an increase in plasma $[\text{HCO}_3^-]$ 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. 1A). That $[\text{HCO}_3^-]$ was unchanged despite a large increase in PCO_2 indicates net acid excretion must have occurred from the intracellular to the extracellular compartment, which is a characteristic of preferential pH_i regulation (Harter et al. 2014; Shartau et al. 2016a). The RBCs 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 buffer value similar to tissues such as the heart [e.g. (fish Wood and LeMoigne 1991) and (reptiles Blomberg and Baldwin 1991; Jensen et al. 1998)] 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 or could 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 H_13 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 not at 90% of incubation (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, at higher 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 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 (Michaelidis et al. 2005; Baker and Brauner 2012; Stapp et al. 2015) and that the response differs between tissues and organisms (Stapp et al. 2015). During hypercarbia in the peanut worm (*Sipunculus nudus* L., 1766), 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; 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 related to the use of acid-base transporters that remain relatively unaffected by pH_e reductions.

Embryonic *A. mississippiensis*, like that of embryonic turtles in Shartau et al. (2016b), 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, submergence of similar stage embryonic chickens for 1 h created hypercarbic hypoxia conditions resulting in a pH_e reduction of ~ 0.8 pH unit and an 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.

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. *Chelydra serpentina* and *A. mississippiensis* embryos can preferentially regulate pH_i even though the adults of these species use coupled pH regulation (Wasser et al. 1991; Shartau et al. 2016a). This 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. 2).

These results show that embryonic *A. mississippiensis* fully protect pH_i against severe reduction in pH_e during hypercarbic 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. that preferential pH_i regulation is an embryonic strategy of acid-base regulation in vertebrates (Shartau et al. 2016a), 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.

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425 **List of symbols and abbreviations**

426

427 H13 Hypercarbic hypoxia (13 kPa PCO_2 , 9 kPa PO_2)

428 kPa Unit of pressure

429 PCO_2 Partial pressure of CO_2

430 pH_e Extracellular blood pH

431 pH_i Intracellular tissue pH

432 PO_2 Partial pressure of O_2

433 RBC Red Blood Cell

434

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Figure legends

Figure 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 $[\text{HCO}_3^-]$ are presented on a $\text{pH}-\text{HCO}_3^-$ 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 for chicken embryos (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 $\pm$ 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 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% of incubation) and *Chelydra serpentina* (snapping turtle; 70 and 90% of incubation hatch), and in post-hatch *C. serpentina*. 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* (Shartau et al. 2016b). Differences are shown as means only to visualize the changes across developmental ages. Values that are

458 ≥ 0 are representative of preferential pH_i regulation while those that are < 0 indicate a net
459 acidosis.

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Change in pH from control