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Short communication

Temperature effects on blood gases in embryonic American alligators (*Alligator mississippiensis*)

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ABSTRACT

Numerous studies report on the influence of temperature on blood gases in ectothermic vertebrates, but there is merely a cursory understanding of these effects in developing animals. Animals that develop in eggs are at the mercy of environmental temperature and are expected to lack the capacity to regulate gas exchange and may regulate blood gases by means of altered conductance for gas exchange. We, therefore, devised a series of studies to characterize the developmental changes in blood gases when embryonic alligators were exposed to 25, 30 and 35 °C. To determine how blood parameters were impacted by changes in embryonic temperature, blood was sampled from the chorioallantoic membrane artery. The blood in the chorioallantoic membrane artery is a mixture of oxygen-poor and oxygen-rich blood, which based on the embryonic vascular anatomy may reflect blood that perfuses the chemoreceptors of the developing animal. Our findings indicate that following a 48 h exposure to 25 °C or 35 °C, there was a positive relationship between CAM artery blood PO_2, PCO_2 and glucose. However, blood pH suggests embryonic alligators lack an acute regulatory mechanism for adjusting blood pH.

1. Introduction

Most embryos of reptiles and many other ectothermic vertebrates are at the mercy of the environment when developing inside an egg. As they are unable to move, these developing animals cannot avoid environmental changes and homeostasis must therefore be maintained solely by physiological means. Crocodilians typically lay their eggs in a nest with suitable abiotic conditions and display some degree of parental care, but the developing eggs will nevertheless experience fluctuations in temperature, as well as hypoxia and hypercapnia. For example, American alligators (*Alligator mississppiensis*), experience both short and long term nest temperature changes (Magnusson et al., 1985; Rhodes and Lang, 1996) which can range from 23 to 36 °C during embryonic development (Coulson and Hernandez, 1983). Fluctuating temperatures can impact the morphology and physiology of embryonic reptiles (Ackerman, 1980; Magnusson et al., 1985; Packard et al., 1985; Rhodes and Lang, 1996) and short-term changes in incubation temperature affect cardiovascular function in American alligators (Marks et al., 2013; Nelson et al., 2018). While these traits of embryonic development are recognized, the effects of temperature or chronic hypoxia on embryonic blood parameters in reptiles is unknown.

It is well established that ectothermic vertebrates reduce arterial pH (pHa) when temperature increases through an elevation of arterial PCO_2 ($PaCO_2$) (Burton, 2002; Glass and Soncini, 1995; Wang and Jackson, 2016). This is a regulated response that is brought about through a reduction in ventilation relative to CO_2 production (VCO_2), such that $PaCO_2$ increases with temperature (Burton, 2002; Glass and Soncini, 1995; Wang and Jackson, 2016). While hypotheses, such the alpha-stat hypothesis, proposes that the reduction in pHa serves to maintain protein ionization and function (Reeves, 1972), the regulated variable and the functional implications remain discussed. Nevertheless, the reduction in pHa with increased temperature is ubiquitous amongst ectothermic vertebrates with very few exceptions such as varanid lizards (Wood et al., 1977a; Wood et al., 1977b). Embryonic animals, enclosed in the egg, represent an interesting condition in the context of temperature-dependent acid-base regulation because the ability to regulate CO_2 excretion is likely to be limited and largely determined by the conductance over the shell and embryonic membranes. Thus, they

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are like lungless salamanders where $PaCO_2$ increases with temperature because metabolic CO_2 production increases more than the conductance for cutaneous CO_2 excretion (Moalli et al., 1981). We would therefore expect that blood PCO_2 increases with temperature in the embryonic alligators, and that pH is likely to decrease.

Our goal was to study the effect of temperature on blood gases of an embryonic reptile, which lacks the capacity to alter $PaCO_2$ through pulmonary ventilation and therefore cannot acutely alter the conditions for CO_2 excretion. Our investigation was designed to further the understanding of how temperature impacts blood parameters in embryonic alligators which utilize the chorioallantic membrane (CAM) as the gas exchange structure. Blood was sampled from the CAM artery because blood in this vessel represents the mixture of arterial and venous blood. Our prediction was that 48 h of exposure to temperature increases or decrease would result in a negative correlation in pH vs temperature.

2. Materials and methods

2.1. Egg collection and staging

American alligator eggs (*Alligator mississippiensis*) were collected in the Rockefeller Wildlife Refuge in Grand Chenier (LA, USA) and transported to the University of North Texas in Denton (TX, USA). At approximately 20 % of incubation 125 eggs were divided into experimental groups used in the present study (Ferguson, 1985).

2.2. Incubation conditions

Eggs were incubated in 1 L plastic Ziploc© boxes with a 1:1 mixture of vermiculite and water at 30 °C to ensure that all embryos developed as females, and water was added when needed to maintain water content of the vermiculite. The two groups consisted of 59 eggs that were incubated in normoxia (21 % O_2) and 66 eggs that were in hypoxia (10 % O_2), where the later value resemble values from crocodilian nests (Lutz and Dunbar-Cooper, 1984). The O_2 mixtures were maintained with air pumps and compressed nitrogen tanks (Eme et al., 2011; Galli et al., 2016) and gas composition was monitored continuously with an oxygen analyzer (S-3AI, Applied Electrochemistry, Berwyn, PA).

2.3. Temperature manipulation

At 70 % and 90 % of incubation, eggs from both O_2 regimes were moved to chambers at either 25 °C, 30 °C or 35 °C for 48 h. These temperatures were maintained by placing the eggs on cotton wool in one of 18 water-jacketed 700 cm^3 chambers (7x7x10cm), surrounded by a 1-in. water-jacketed space with a continuous water supply from a Polestar® temperature-controlled circulator (Cole Parmer, Court Vernon Hills, IL). Humidified air was continuously pumped into each chamber at ~0.2 $L{\cdot}min^{-1}$. Each chamber was sealed using a lid with ports for the catheter, a thermocouple, and the airline. The animals were maintained at either temperature for 48 h before blood was sampled for analysis of blood gases.

2.4. Blood sampling from the CAM vasculature

To sample blood, the eggs were candled to locate a CAM artery, and placed in a temperature-controlled surgical chamber, where a portion of the eggshell could be removed under a dissection microscope (Leica MZ6; Leica Microsystems, Waukegan, IL). A heat-pulled PE 50 tubing filled with heparinized saline (0.9 % NaCl, 50 units ml^{-1}) was inserted in the CAM artery and forward into the major artery leaving the embryo (Crossley II and Altimiras, 2005; Crossley II et al., 2003) whereupon a 500 ul blood sample was drawn into a heparinized syringe as well as two 50 ul heparinized microhematocrit tubes (Scientific Products, MaGaw Park, IL) that were then sealed with clay for blood analysis.

2.5. Measurements of blood gases

All blood samples were immediately analyzed for PO_2, PCO_2, and pH using a Radiometer BMS MK 2 Blood Micro-System (Radiometer, Copenhagen, DK) with the electrodes maintained at the same temperature as the eggs using a circulating water bath (RM 6 RMA, LAUDA-Brinkmann LP, Delran, NJ). Oxygen content was determined as described by Tucker (Tucker, 1967) and total CO_2 content of the plasma and whole blood according to Cameron (Cameron, 1971), and plasma bicarbonate values were calculated as previously described (Austin et al., 1963; Busk et al., 2000; Jensen et al., 1998). Hematocrit was measured after centrifugation (20,854 *g* for 5 min at Micro-Hematocrit Damon/IEC Division, MA). Whole blood glucose and lactate concentrations were measured with a glucose and lactate analyzer (2300 STAT Plus, YSI, Yellow Springs, OH). Plasma osmolarity was measured with a vapor pressure osmometer (Vapro Model 5600, Wescor Logan, UT).

2.6. Statistical analysis

All measurements were analyzed with a MANOVA (Statistica v13; StatSoft, Tulsa, OK) with incubation percentage, incubation oxygen levels and exposure temperature used as the independent variables. Newman Kuels (NK) post hoc tests were used to identify specific differences in each parameter between the independent variables. Our initial analysis demonstrated that incubation oxygen levels did not influence any of the measured parameters and data for both incubation oxygen levels were therefore combined and analyzed using the tests outlined with incubation percentage and exposure temperature used as the independent variables. Data are presented as means ± SE, with statistical significance being designated when $p \leq 0.05$.

3. Results and discussion

The partial pressure of oxygen in the CAM artery ($P_{CAM}O_2$) increased significantly with temperature in both developmental stages (Fig. 1A) and was significantly lower at 90 % of incubation at 35 °C compared to the 70 % embryos (Fig. 1A). The oxygen content in the CAM artery was similar at both temperatures and developmental stages (Fig. 1B) and resembled measurements in our recent study at 30 °C (Crossley 2nd et al., 2024). pH in the CAM artery changed significantly with temperature at both developmental stages and there was a significant interaction with temperature as the pH changed differently with temperature as the embryos matured (Fig. 1C). Thus, it was only at 90 % of incubation that CAM artery pH decreased consistently as temperature rose (Fig. 1C). Nevertheless, at both developmental stages there was a clear rise in the partial pressure of CO_2 in the CAM artery ($P_{CAM}CO_2$) as temperature increased (Fig. 1D) and $P_{CAM}CO_2$ was consistently higher at 70 % compared to 90 % development. Plasma bicarbonate $[HCO_3^-]_{pl}$ significantly differed with developmental stage and temperature (Fig. 1E). Temperature significantly impacted hematocrit at 70 % of incubation, with values that were significantly lower at 30 °C compared to the other two temperatures (Fig. 1F). At 90 % of incubation, temperature had no effect on hematocrit (Fig. 1 F). Whole blood lactate concentration [Lac] was significantly higher in embryos at 90 % of incubation. Whole blood glucose concentration [Glucose] was similar at both developmental stages (Table 1) but differed significantly with temperature. Plasma osmolarity was affected by temperature, developmental stage with an interaction of temperature.

$P_{CAM}O_2$ in embryonic alligators of our study were similar to those reported for embryonic birds at similar points in incubation (Carey et al., 1993; Girard, 1971; Tazawa, 1980; Tazawa and Mochizuki, 1978) and embryonic crocodilians (Grigg et al., 1993). There were noted differences in how the changes in temperature impacted arterial blood PO_2 between the developmental percentages studied, with increases in PO_2 with each successive increase in temperature at 70 % of incubation while there was only an increase at 90 % when temperature was

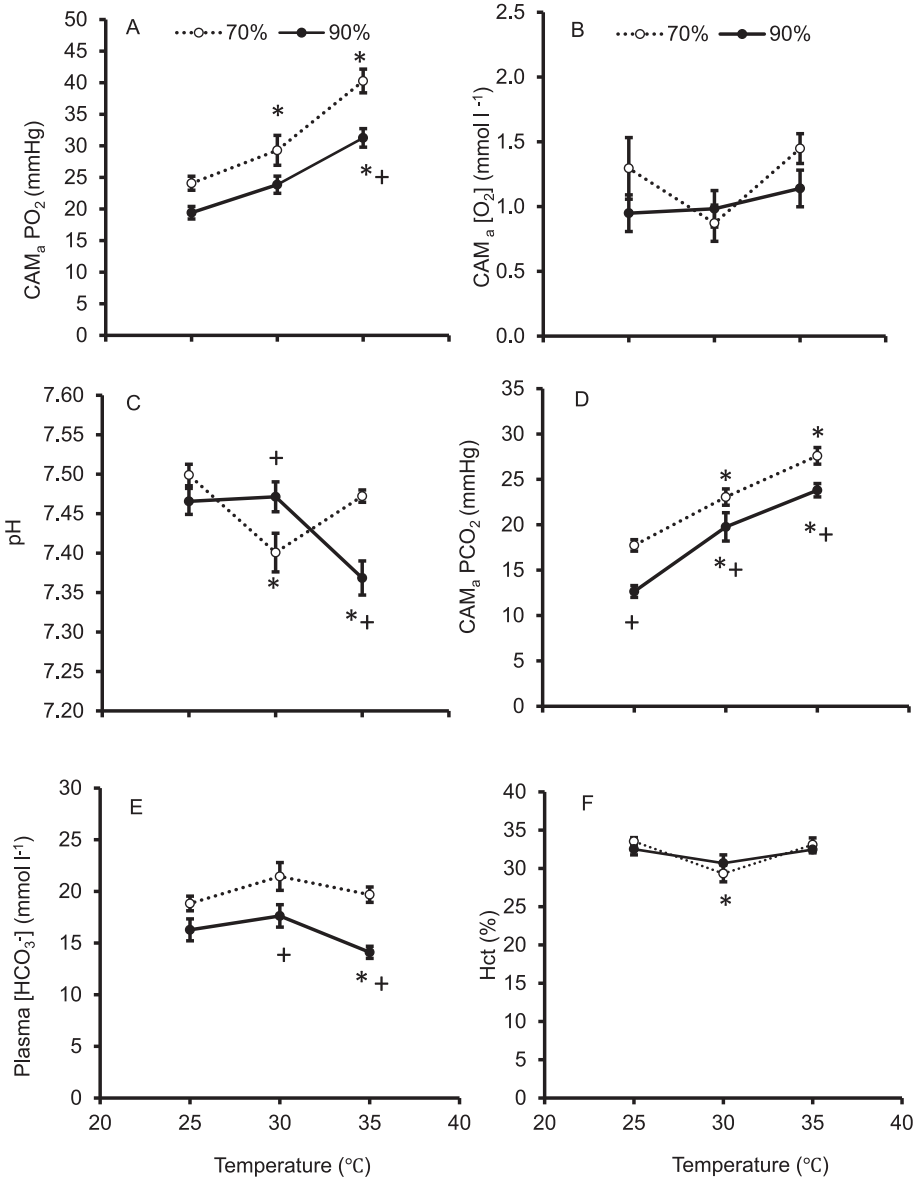

Fig. 1. CAM arterial PO_2 (A), oxygen content $[O_2]$ (B), pH (C), PCO_2 (D), plasma bicarbonate $[HCO_3^-]_{pl}$ (E) and hematocrit (Hct) (F) from embryos at 70 % (open circles and dashed line) and 90% (filled circles and solid line) of incubation subjected to 48 h. of 25 °C, 30 °C or 35 °C. A single asterisk indicates significant differences from the values at the lower temperature. A + symbol indicates a significant difference between the incubation ages within a given temperature. Data are presented as mean ± SEM. Sample sizes are given in Table 1.

increased to 35 °C (Fig. 1A). Further at 90 % of incubation PO_2 was lower at 35 °C compared to 70 % of incubation (Fig. 1A). Increasing temperature would be predicted to increase metabolic rate of the developing animals as it does in juvenile alligators (Lewis and Gatten, 1985). In juvenile alligators increasing temperature over the same range as in our study resulted in a positive correlation in standard metabolic rate (Lewis and Gatten, 1985). The increased embryonic CAM arterial blood PO_2 at 70 % of development may therefore be related to increases in CAM vessel reactive hyperemia resulting in an increase in surface area for gas diffusion. Irrespective of the changes in CAM artery PO_2, blood O_2 content was similar across all temperatures, and developmental percentages indicating the PO_2 differences were insufficient to change the degree of hemoglobin saturation in embryonic alligators. This indicates that the increase in embryonic metabolic rate which would be predicted with increasing temperature is matched by increases in cardiac output of the embryonic alligator. Prior studies have demonstrated that embryonic alligators increase heart rate with increases in temperature (Marks et al., 2013; Nelson et al., 2018) suggesting that any temperature associated increases in metabolic rate is supported by increases in cardiac output of the embryonic alligator.

The effects of temperature on blood acid base state in ectotherms has been addressed numerous times over the past century (Austin et al., 1927; Glass et al., 1985; Reeves, 1972; Stinner and Hartzler, 2000; Wang and Jackson, 2016). In general, there is an inverse relationship between body temperature of air breathing ectotherms where blood pH decreases with increasing body temperature at a constant value of −0.016 U/°C (Burton, 2002; Howell et al., 1970). Further, as temperature decreases ectothermic species adjust ventilation increasing $[HCO_3^-]$ while PCO_2 decreases (Cameron, 1989). However, egg laying terrestrial vertebrates lack the ability to adjust ventilation during periods of pH disturbance that accompany changes in ambient temperature. Based on these prior studies we first predicted that blood pH would decrease with increasing temperature in this study. However, this was not the case in the embryonic alligators. We did, however, observe the expected rise in in PCO_2 in the CAM artery, which is likely to reflect that the rise in metabolic CO_2 production that resulted from the rise in temperature that

Table 1
Values for whole blood lactate concentration (Lactate) mmol l^{-1}, glucose concentration (Glucose) mmol l^{-1} and blood plasma osmotic concentration (Osmo) for embryonic alligators at 70 % and 90 % of incubation (Inc) after 48 h exposure to 25, 30, or 35 °C. A single asterisk represents a different value than those in embryos at the other two temperatures. A + symbol indicates a significant difference between the incubation ages within a given temperature. A double asterisk represents differences from values in embryos at 30 °C only. Data are presented as mean ± sem. Sample sizes are given in parentheses.

Incubation (%)	Measurement	25 °C	30 °C	35 °C
70	Lactate (mmol l^{-1})	0.5 ± 0.1 (16)+	1.0 ± 0.2 (17)	0.9 ± 0.1 (15)
90	Lactate (mmol l^{-1})	1.2 ± 0.2 (13)	1.2 ± 0.2 (15)	1.6 ± 0.3 (16)
70	Glucose (mmol l^{-1})	1.9 ± 0.2*	3.0 ± 0.2	3.7 ± 0.2
90	Glucose (mmol l^{-1})	1.7 ± 0.2*	3.1 ± 0.1	3.7 ± 0.3
70	Osmo (mmol l^{-1})	281 ± 3**	273 ± 2	272 ± 2
90	Osmo (mmol l^{-1})	293 ± 2+	281.0 ± 3*	293 ± 2+

was not attended by a proportional rise in CO_2 conductance across the shell. This is similar to the skin-breathing salamander (*Cryptobranchus alleganiensis*) where arterial PCO_2 increases with increased temperature (Moalli et al., 1981). However, in contrast to the hellbender, where pH fell as predicted by the alpha-stat hypothesis (Moalli et al., 1981), we did not observe a constitutive reduction in pH of the CAM artery blood when temperature rose in the alligator embryos.

At 70 % of incubation, our measurements resemble values reported for embryonic chickens at a similar developmental time points (Black and Burggren, 2004; Boutilier et al., 1977; Girard, 1971; Piiper et al., 1980; Tazawa and Mochizuki, 1978; Willemsen et al., 2011). Further, pH values at 30 °C were similar to those previously reported for embryonic alligator at 70 % of incubation (Crossley 2nd et al., 2024). In response to 48 h at 25 °C blood pH increased relative to embryos at 30 °C (Fig. 1C). Importantly, blood PCO_2 was lower at 25 °C compared to 30 °C as would be predicted with an increase in blood pH (Fig. 1D). Interestingly, plasma HCO_3^- was constant regardless of the pH or PCO_2 of the blood (Fig. 1E). This suggests that embryonic alligators at 70 % of incubation experienced a relative uncompensated respiratory alkalosis at 25 °C relative to those embryos at 30 °C. In response to 48 h at 35 °C, blood pH was similar to values at 30 °C (Fig. 1C). In this case the relatively constant blood pH was accompanied by an increase in blood PCO_2 (Fig. 1C and D). Again, increasing temperature to 35 °C had no effect on HCO_3^- compared to 30 °C (Fig. 1E). The relative constant blood pH while PCO_2 increases without changes in HCO_3^- in 70 % of incubation embryos is difficult to explain. This pattern differs from those that would be predicted for airbreathing vertebrates. While speculative, the data indicates other blood buffering systems must have offset the predicted decrease in pH with increases in PCO_2 in embryonic alligators at 70 % of incubation.

At 90 % of incubation, blood pH at 30 °C was similar to that previously reported for embryonic alligators at this point of incubation (Shartau II et al., 2018). pH values in this study were lower than those reported for the estuarine crocodile, however incubation temperatures were not included in the prior study, which may be the basis for the difference (Grigg et al., 1993). Interestingly 48 h at 25 °C had no effect on blood pH nor HCO_3^- but PCO_2 did decrease (Fig. 1C, D, and E). Given that PCO_2 decreased without changes in the other two parameters suggests again that at 90 % of development an additional buffering mechanism maybe present in embryonic alligators. After 48 h at 35 °C CAM arterial blood pH decreased however, blood PCO_2 increased and plasma HCO_3^- decreased (Fig. 1C, D and E). The decrease in blood pH with increased temperature indicates an uncompensated acidosis. Given our findings, we suggest there may be a metabolic acidosis at 90 % of incubation however further investigations are needed to clarify this speculation.

Blood lactate at 70 % and 90 % of development as similar to those previously reported for embryonic chickens incubated at different temperatures (Willemsen et al., 2011) and similar to those reported for embryonic alligators incubated at 30 °C (Crossley 2nd et al., 2024). At each time point of incubation, blood lactate concentration was unaffected by temperature however there was a developmental percentage effect (Table 1). Specifically lactate concentration was lower in embryos at 70 % of incubation, compared to 90 % of incubation after 48 h at 25 °C (Table 1). This suggests decreasing temperature, reduces O_2 delivery in older embryos which could be attributed to the reduction in heart rate known to accompany decreases in egg temperature (Marks et al., 2013; Nelson et al., 2018). Unlike blood lactate, glucose concentration in most cases showed a positive relationship with temperature (Table 1). The general pattern was similar to that reported for embryonic chickens subjected to a range of temperatures during incubation (Willemsen et al., 2011). After 48 h at 25 °C glucose concentration was lowest at both developmental time points studied (Table 1). A prior study of embryonic chicken suggested the increase in blood glucose could be attributed to an increase in gluconeogenesis with higher temperatures (Willemsen et al., 2011). In embryonic alligators at 25 °C blood glucose concentration was significantly lower than that at 30 °C (Table 1). Again, a decrease in the rate of gluconeogenesis with decreased temperatures could account for the reduced blood glucose levels. The glucose data combined with the lactate increases at low temperature in embryonic alligators suggests a shift from aerobic metabolism to anaerobic metabolism as embryonic alligators age.

In summary, our use of temperature was an attempt to test the embryos capacity for acid base regulation given the known relationship between temperature and blood pH in ectothermic vertebrates. While our findings are novel, they do not support our initial predictions. It is important to note that the timeframe of 48 h exposure to the test temperatures may have been insufficient to significantly alter metrics of acid base balance. Future studies should incorporate chronic incubation at the temperatures used in this study.

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CRediT authorship contribution statement

Dane A. Crossley: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Janna L. Crossley:** Writing – original draft, Methodology, Investigation, Data curation. **Justin L. Conner:** Writing – original draft, Methodology, Investigation, Formal analysis. **Brandt Smith:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Ruth Elsey:** Writing – original draft, Methodology. **Derek Nelson:** Methodology. **Tobias Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare to have no known competing financial interests or personal relationships that could have appeared to influence the data in this manuscript.

Data availability

Data will be made available on request.

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