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Short communication

Short communication: Characterizing arterial and venous blood gases over the gas exchange surface, the chorioallantoic membrane, of embryonic American alligators (*Alligator mississippiensis*) at two points of development

Dane A. Crossley II [a,*], Janna L. Crossley [a], Justin L. Conner [a], Brandt Smith [a], Ruth Elsey [b], Derek Nelson [a], Tobias Wang [c]

[a] Department of Biological Sciences, University of North Texas, Denton, TX, USA
[b] Department of Wildlife and Fisheries, Grand Chenier Louisiana, LA, USA
[c] Zoophysiology, Department of Bioscience, Aarhus University, Denmark

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ABSTRACT

Assessments of arterial and venous blood gases are required to understand the function of respiratory organs in animals at different stages of development. We measured blood gases in the arteries entering and veins leaving the chorioallantoic membrane (CAM) in embryonic alligators (*Alligator mississippiensis*). The CAM accounts for virtually all gas exchange in these animals, and we hypothesized that the CAM vasculature would be larger in eggs incubated in hypoxia (10% O_2 for 50% or 70% of incubation), which would be reflected in a lower partial pressure of CO_2 (PCO_2). Contrary to this hypothesis, our measurements revealed no effects of hypoxic incubation on PCO_2, and seemingly no increase in vascularization of the CAM in response to incubation in 10% O_2. PCO_2 was lower on the venous side, but only significantly different from arterial blood at 70% of incubation. The calculated blood flow to the CAM increased with development and was lower in both groups of alligators that had been incubated in hypoxia. Future studies should include measurements of blood parameters taken from embryos held in conditions that mirror incubation O_2 levels, in combination with direct measurements of CAM artery blood flow.

Embryonic metabolism relies on adequate convective transport of respiratory gases and nutrients as well as removal of metabolic waste products by the cardiovascular system to complete normal development and organogenesis. Although functional and morphological development of the cardiovascular system has been studied in some reptilian embryos, little is known about blood gas composition and levels of metabolites in the arterial and venous circulation in developing reptiles. This type of data is required to understand how hypoxia can affect organ developmental trajectory and embryonic O_2 demand during development (Crossley II et al., 2017; Wearing et al., 2017; Joyce et al., 2018). Therefore, we measured partial pressures of CO_2 and O_2 (PCO_2 and PO_2, respectively) as well as: pH, lactate, glucose, and whole blood bicarbonate concentration of the chorioallantoic membrane (CAM) arterial and venous blood in embryonic alligators at approximately 70% (~70%) and approximately 90% (~90%) of incubation; that is, at 50 and 65 days, respectively, of a 72-day incubation period at 30 °C subjected to either 21% O_2 (the N21 group) or 10% O_2 (the H10 group) during incubation. The CAM serves as the gas exchange structure in embryonic alligators, with arterial blood consists of a mixture of oxygen-rich and oxygen-poor blood, while the venous blood leaving the CAM carries oxygen-rich blood. The anatomy of the CAM vasculature in egg laying amniotic vertebrates has been described and reviewed in earlier studies (*e.g.* Tazawa and Mochizuki, 1977; Burggren and Rojas Antich, 2020). The goal of this study was to characterize embryonic convective transport and to determine the effects of chronic hypoxia.

We sampled blood from the CAM artery (mixed venous blood) or the vein leaving the CAM (arterialized blood) in eggs (sample size given in Table 1) from 10 clutches of American alligator eggs collected from wild nests on the Rockefeller Wildlife Refuge (Grand Chenier, LA) and transported to University of North Texas. Eggs were then handled and O_2 incubation conditions were maintained and monitored as described by Smith et al. (2019). Briefly, two eggs from each clutch were sampled to determine embryonic age. Eggs at approximately 20% of incubation were then placed in either 21% O_2 or 10% O_2 in an environmental

* Corresponding author.
E-mail address: dane.crossley@unt.edu (D.A. Crossley).

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Table 1
Values for hematocrit (Hct), and blood plasma osmotic concentration (Osm) for embryonic alligators incubated in normoxia (N21) or 10% O_2 (H10) conditions (Cond) at ~70% and ~ 90% of incubation of blood taken from the CAM artery or vein. An asterisk indicates a difference within incubation percentage and condition between blood sample sites. Dissimilar letters indicate differences in blood values from samples taken from the CAM artery or vein across incubation percentages and conditions. Data are presented as mean ± SEM.

Incubation (%) & Condition	Site (N)	Osm (mmol l^{-1})	Hct (%)
~70% H10	CAM Artery (7)	272 ± 3*	26.0 ± 1.2*^{A}
~70% H10	CAM Vein (6)	264 ± 2 ^{A}	30.0 ± 0.7
~70% N21	CAM Artery(11)	275 ± 3*	31.1 ± 1.2^{B}
~70% N21	CAM Vein (6)	270 ± 5 ^{A}	31.5 ± 0.5
~90% H10	CAM Artery (8)	281 ± 3*	29.6 ± 1.6^{B}
~90% H10	CAM Vein (7)	294 ± 2^{B}	32.7 ± 1.9
~90% N21	CAM Artery (7)	281 ± 6*	32 ± 1.5^{AB}
~90% N21	CAM Vein (7)	295 ± 3^{B}	30.6 ± 0.7

chamber at 30 °C. Both oxygen levels were maintained by placing boxes containing the eggs in large sealed plastic bags. Gas composition within each bag was maintained by a continuous gas flow, and oxygen levels were monitored continuously as previously described (Smith et al., 2019). At either ~70% or ~ 90% of the 72-day incubation at 30 °C, eggs were instrumented with a catheter in either a tertiary CAM artery or vein (Crossley II and Altimiras, 2005). The cannulations were conducted in a customized temperature control chamber at 30 °C and blood samples of approximately 500 ul were drawn from the respective blood vessel.

We determined PCO_2, PO_2, and pH using a Radiometer BMS MK 2 Blood Micro-System (Radiometer, Copenhagen, DK) with the electrodes kept at 30 °C with a circulating water bath (RM 6 RMA, LAUDA-Brinkmann LP, Delran, NJ). We measured O_2 concentration, [O_2], as described by Tucker (Tucker, 1967), and total CO_2 content of whole blood was determined according to Cameron (Cameron, 1971). Total CO_2 content was then used to determine whole blood bicarbonate which was calculated as previously described (Busk et al., 2000). 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

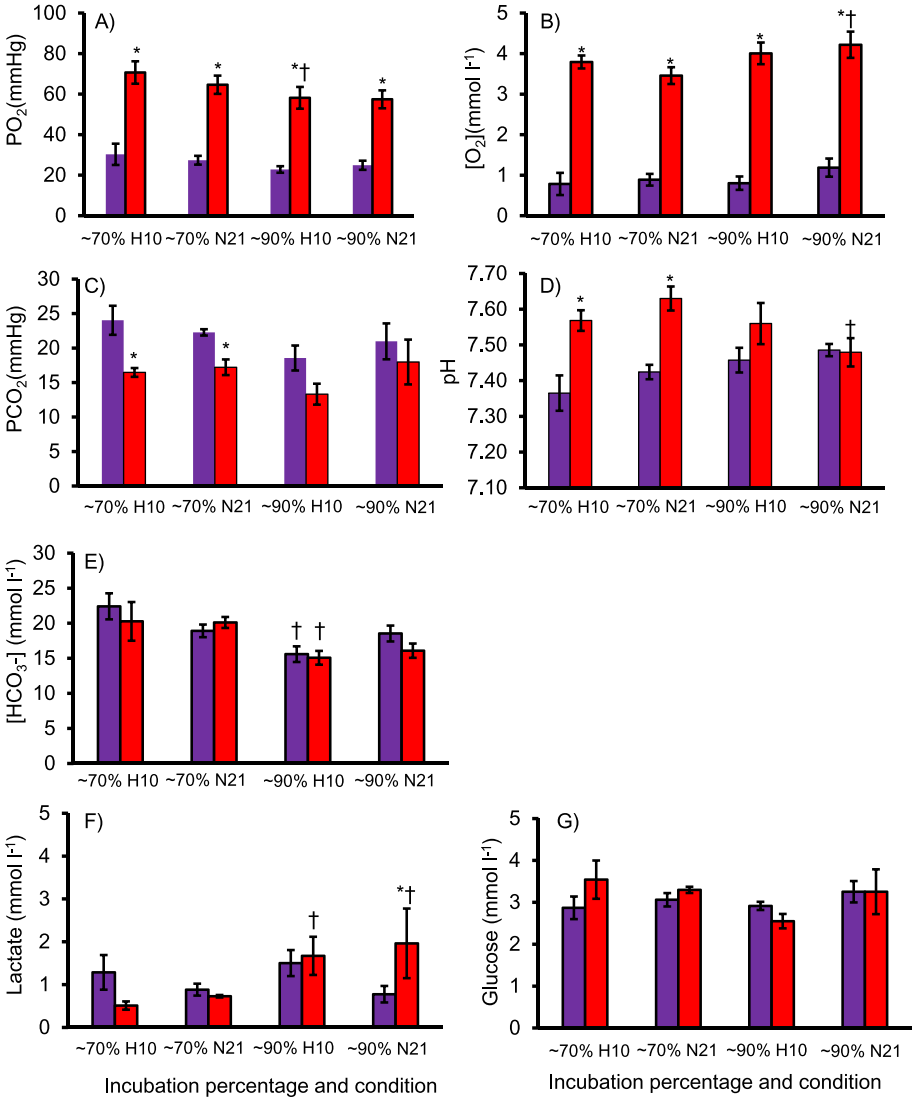

Fig. 1. CAM artery (purple column) and vein (red column) PO_2 (A), oxygen concentration (B), [O_2], PCO_2 (C), pH (D), whole blood bicarbonate [HCO_3^-] (E), whole blood lactate concentration [Lac] mmol l^{-1} (F) and glucose concentration [Glucose] mmol l^{-1} (G) of embryos incubated in normoxic (N21) and hypoxia (H10) sampled at ~70% and ~ 90% of incubation, respectively. An asterisk indicates significant differences between the CAM artery and vein within incubation condition and percentage. A dagger indicates significant differences between incubation percentages within incubation condition for a given vessel. Data are presented as mean ± SEM. Sample sizes are given in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyzer (2300 STAT Plus, YSI, Yellow Springs, OH). Plasma osmolarity was measured with a vapor pressure osmometer (Vapro Model 5600 Wescor, Logan, UT).

Blood flow to the CAM index ($\dot{Q}_{index}$) was calculated using the Fick equation ($\dot{V}O_2 = \dot{Q}_{index} \bullet A\text{-}V_{O2}$; Sartori et al., 2018), using the $A\text{-}V_{O2}$ differences from the present study and O_2 consumption ($\dot{V}O_2$) values previously published (Crossley II et al., 2017). The calculated total $\dot{Q}_{index}$ per embryo was then multiplied by the embryonic mass values at ~70% of incubation for H10 of 14.0 ± 0.7 g and N21 of 18.9 ± 0.7 g and at ~90% of incubation for H10 of 21.9 ± 0.7 g and N21 of 37.0 ± 2.6 g which were the masses of each group of embryos in our study.

All blood measurements were analyzed using a MANOVA with incubation percentage, incubation condition, and sample vessel as the independent variables. A Fisher's LSD *post hoc* test was used to identify specific differences in each parameter. Data are presented as means ± SE, with statistical significance designated as $p \leq 0.05$.

The PO_2 was significantly higher in the CAM vein compared to the artery (Fig. 1A). Further, CAM artery PO_2 was similar across incubation conditions and percentages (Fig. 1A). The only significant difference was between CAM venous blood in the H10 groups at ~70% and ~ 90% of incubation, with a decrease of approximately 18% in PO_2 at ~90% of incubation (Fig. 1A). A similar pattern was found for blood $[O_2]$: CAM venous $[O_2]$ was significantly higher than arterial blood. There were no differences in CAM arterial blood $[O_2]$ between incubation conditions or percentages (Fig. 1B); CAM venous blood $[O_2]$ was similar between incubation conditions within each percentage of incubation; however, levels were significantly higher by approximately 19% in the N21 group at ~90% of incubation compared to ~70% (Fig. 1B). This difference in CAM vein blood $[O_2]$ was not due to changes in Hct (Table 1). CAM artery PCO_2 was similar across incubation conditions and incubation percentages (Fig. 1C). In embryos at ~70%, venous PCO_2 was lower than arterial blood values; this was not the case at ~90% of incubation. Blood pH showed a similar yet inverted pattern with no difference in CAM artery blood pH across incubation percentages and conditions (Fig. 1D). At ~70% of incubation, CAM venous blood pH was significantly greater than CAM arterial blood pH. At ~90%, there was no difference in blood pH between CAM arterial and venous blood (Fig. 1D). Further, there was no difference in CAM venous blood pH within incubation percentage between the N21 and H10 groups. However, pH of CAM venous blood in N21 embryos was higher at ~70% of incubation than at ~90% of incubation (Fig. 1D). There was no difference in whole blood $[HCO_3\text{-}]$ independent of the incubation condition, percentage, or blood sample site. The notable exception was that blood taken from the CAM vein and artery of H10 embryos at ~90% of incubation, which had significantly lower values than at ~70% of incubation (Fig. 1E). We also found limited differences in blood lactate and glucose values. CAM blood lactate was similar at ~70% of incubation and was independent of incubation conditions or sample site (Fig. 1F). At ~90% of incubation, arterial blood lactate values were similar to those at ~70% of incubation, independent of incubation condition, but there were significant differences in lactate values of blood taken from the CAM vein, with higher values at ~90% than ~70% of incubation (Fig. 1F). Finally, blood glucose was constant and independent of developmental stage, condition, or sample location.

Calculated CAM $\dot{Q}_{index}$ in embryos at ~70% was approximately 1.5 and 1.0 ml min g^{-1} in N21 and H10 groups, respectively, and at ~90% of incubation it was 1.9 and 1.2 ml min g^{-1} in the N21 and H10 groups, respectively.

This study is the first to measure arterial and venous blood differences across the gas exchange surface of embryonic crocodilians incubated in low O_2. Our findings indicate that blood parameters exhibit limited changes as development progresses and possess limited plasticity. Further, calculated CAM $\dot{Q}_{index}$ increased with incubation in both the H10 and N21 experimental embryos; however, CAM $\dot{Q}_{index}$ in the H10 embryos was lower than the N21 embryos at both time points of incubation. Prior studies of embryonic American alligators demonstrated that both morphological and physiological maturation of the cardiovascular system exhibits developmental phenotypic plasticity (Crossley II and Altimiras, 2005; Eme et al., 2011a; Crossley II and Altimiras, 2012; Crossley II et al., 2012; Tate et al., 2016; Crossley II et al., 2017; Alderman et al., 2019). However, as previously demonstrated for embryonic alligator hemoglobin, blood parameters are relatively unaffected by hypoxic incubation (Bautista et al., 2021).

The PO_2 of CAM artery blood of embryonic alligators was similar to that reported for embryonic estuarine crocodiles at the end of the incubation period, although CAM vein blood PO_2 was higher in the alligator embryo at both points of incubation compared to the crocodile (Grigg et al., 1993). There was no difference in CAM vein blood PO_2 within each incubation percentage between H10 and N21 embryos (Fig. 1A). A prior study found that regional hypoxia, induced by coating the eggshell with wax, caused CAM hypervascularization in embryonic alligators (Corona and Warburton, 2000). If CAM hypervascularization occurred in the current study in the H10 embryos, it had no impact on the CAM vein blood PO_2, $[O_2]$, PCO_2, nor pH, as these values were similar in N21 embryos (Figs. 1A-1D). We sampled blood in embryos held at ambient PO_2 and PCO_2, which may be the basis for the lack of measured differences. Either hypervascularization was not induced by chronic incubation in 10% O_2, or the CAM surface area of American alligators is optimized for gas exchange. If hypervascularization had occurred in the embryos we used, it would be predicted that PCO_2 would be lower in the H10 compared to the N21 embryos; however, there was no difference. Therefore, although CAM vascular density was not measured in the current study, the blood parameter data suggest that incubation in 10% O_2 was not sufficient to change the CAM vascular development.

CAM artery and vein blood differences in PCO_2 were only significant at ~70% of incubation, and the AV difference in PCO_2 was almost significant in the H10 embryos at 90% ($p = 0.062$; Fig. 1C). In N21 incubated embryos, mass-specific $\dot{V}CO_2$ decreases as the embryo matures for ~70% to ~90% of incubation (Crossley II et al., 2017). However, when $\dot{V}O_2$ is expressed per egg, $\dot{V}O_2$ increases with incubation. The pH of CAM artery blood exhibited an inverted pattern compared to the PCO_2 pattern, with lower values in artery blood compared to vein blood in the embryos at ~70% of incubation. This would be predicted based on the PCO_2 values (Figs. 1C and D). Surprisingly, there was no difference in $[HCO_{3\_}]$ within each incubation age, conditions, and sample location (Fig. 1E); an exception was a reduction in $[HCO_{3\_}]$ in H10 embryos at ~90% compared to ~70% (Fig. 1E). The basis for this difference is unknown.

Although glucose did not differ with incubation percentage, condition, or sample site, blood lactate increased with age in both groups in the CAM vein (Fig. 1F and G). Lactate concentration in the CAM artery was independent of incubation age or condition (Fig. 1F), suggesting minimal dependence on anaerobic metabolism, as the CAM artery represents a mixture of blood from the tissues and oxygenated blood from the CAM. Conversely, lactate concentration in CAM vein samples increased at ~90% of incubation, and the N21 embryos exhibited a significant increase in CAM vein values compared to CAM artery values (Fig. 1F). While speculative, this suggests the CAM utilizes anerobic metabolism as the embryos develop.

Lastly, the calculations of CAM $\dot{Q}_{index}$, based on published metabolic measurements for embryonic alligators, demonstrate increases in both experimental groups as they transition from ~70% to ~90% of incubation. Further, the H10 embryos have lower CAM $\dot{Q}_{index}$ than the N21 embryos. It should be noted that the direct measures of CAM blood flow previously published were 29 ± 1 ml min^{-1} kg^{-1} for N21 incubated embryos, whereas $\dot{Q}$ CAM was 92 ± 3 ml min^{-1} kg^{-1} for H10 embryos (Eme et al., 2011b). If these values are used to recalculate the CAM blood flow based on the embryonic masses in N21 and H10 embryos at ~70% and ~ 90% of incubation, then blood flow would be 0.5 and 1.3 ml min g^{-1} in the N21 and H10 animals at ~70% of incubation, respectively, and 0.9 and 2.0 ml min g^{-1} in the N21 and H10 animal at

~90% of incubation, respectively. This pattern was markedly different from the calculated values based on the current study, suggesting significant oxygenated/deoxygenated blood mixing occurs prior to blood reaching the CAM.

Our findings suggest that blood parameters change minimally during the ontogenetic window from ~70% to ~90% of incubation. Future studies should focus on measurements in embryos held at their incubation PO_2.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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