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Metabolic responses to chronic hypoxic incubation in embryonic American alligators (*Alligator mississippiensis*)

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ABSTRACT

Chronic hypoxic incubation is a common tool used to study developmental changes in reduced O_2 conditions, and it has been useful for identifying phenotypically plastic periods during ontogeny in laboratory settings. Reptilian embryos can be subjected to natural hypoxia due to nesting strategy, and recent studies have been important in establishing the phenotypic responses of several species to low developmental oxygen. In particular, the cardiovascular responses of American alligators (*Alligator mississippiensis*) to low developmental oxygen have been detailed, including a substantial cardiac enlargement that may support a higher mass specific metabolic rate. However, embryo mass-specific metabolic demands of hypoxic incubated alligator embryos have not been measured. In this study, alligator eggs were incubated in 10% O_2 (H) or 21% O_2 (N) environments for the entire course of embryonic development. Acute metabolic measures in 21% and 10% O_2 were taken for both H and N groups. We hypothesized that acute 10% O_2 exposure has no impact on metabolic rate of embryonic alligators, and that metabolic rate is unaffected by chronic hypoxic incubation when studied in embryos measured at 21% O_2. Our findings suggest phenotypic changes resulting from hypoxic incubation early in incubation, in particular relative cardiac enlargement, enable embryonic alligators to sustain metabolic rate during acute hypoxic exposure.

1. Introduction

Chronic hypoxic incubation is a common tool for investigating physiological and morphological capacities for change during vertebrate development (Siefert et al., 1973; Carlson and Siefert, 1974; Kam, 1993; Warburton et al., 1995; Andrews, 2002; Dzialowski et al., 2002; Crossley and Altimiras, 2005; Nechaeva and Vladimirova, 2008; Flewelling and Parker, 2015; Liang et al., 2015; Smith et al., 2015; Mueller et al., 2015). Low oxygen can be used to assess the capacity for developmental phenotypic plasticity in a laboratory setting, and this abiotic factor is relevant to many species that experience hypoxia as a natural developmental perturbation. Reptilian embryos can be subjected to relative hypoxia, due in part to nesting strategies (Ackerman, 1977; Booth, 1998; Grigg et al., 2010). Crocodilian eggs, like those of many reptiles, incubate in subterranean or mound nests, where microenvironments can vary in gas composition due to factors such as diffusion limitations, precipitation, and increasing embryonic metabolism (Lutz and Dunbar-Cooper, 1984; Packard et al., 1985; Ackerman and Lott, 2004; Grigg et al., 2010), and oxygen values within reptile nests as low as 11–15% have been recorded (Lutz and Dunbar-Cooper, 1984; Miller, 2008). These biotic and abiotic factors directly impact embryonic development (Wallace et al., 2004), and reptilian embryos subjected to chronic hypoxic incubation are physiologically and morphologically impacted, including marked changes in cardiovascular system development (Kam, 1993; Corona and Warburton, 2000; Nechaeva et al., 2007; Nechaeva and Vladimirova, 2008; Owerkowicz et al., 2009; Nechaeva, 2011; Eme et al., 2013b; Flewelling and Parker, 2015).

Our recent work has shown that the cardiovascular systems of American alligators (*Alligator mississippiensis*) and Common snapping turtles (*Chelydra serpentina*) are both altered by hypoxia during embryogenesis, and that each species shows a relative increase in heart mass following chronic hypoxia (Crossley and Altimiras, 2005; Eme et al., 2011a, 2011c, 2013a; Marks et al., 2013; Tate et al., 2015). In American alligators, cardiovascular phenotypic changes are dependent on hypoxic exposure during a relatively brief window of susceptibility beginning at 20% of incubation and continuing for 7–14 days of a ~72-day incubation period (Tate et al., 2016). Cardiac enlargement has been speculated to correlate with an increase in cardiac output to maintain metabolic function of the embryo (Kam,

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1993; Crossley and Altimiras, 2005; Eme et al., 2011a, 2011c, 2012b, 2013a, 2013a, 2014; Tate et al., 2012, 2015; Marks et al. 2013). However, the consequences of chronic hypoxia on metabolic function in embryonic American alligator remain unresolved. To begin to address this question, repeated measures of metabolic function in embryonic animals, in both normoxic and hypoxic conditions must be performed.

A number of studies have investigated metabolic function in crocodilian and turtle eggs incubated under normoxic conditions (Thompson, 1989; Kam, 1993; Kam and Lillywhite, 1994; Birchard and Reiber, 1995; Warburton et al., 1995; Booth, 2000; Grigg et al., 2010), however, measures of mass specific metabolic function of embryos studied in hypoxia are more laborious and have yet to be completed. Repeated measures of embryonic American alligator metabolism (not corrected for embryo mass) have demonstrated mildly hypoxic (17% O_2) and normoxic-incubated (21% O_2) eggs were virtually identical until hatch time (Warburton et al., 1995). It is important to note the while "egg" metabolic measures are valuable, they could contain inherent error due to variable yolk or embryo size between normoxic and hypoxic-incubated animals. Embryo-mass specific metabolic rate could reveal important energy usage during critical windows of development, and these measures are essential to understanding the metabolic cost of altered developmental environments (Mueller et al., 2015). Prior studies have noted that embryonic alligators incubated in 10% O_2 maintained similar heart rates to control animals until 90% of incubation, after which heart rate was depressed (relative bradycardia) in 10% O_2–incubated alligator embryos, possibly representing a depression in metabolic function. Further, the known bradycardic response to acute hypoxia at 70% and 90% of incubation is blunted in 10% O_2–incubated alligators, also suggesting altered metabolic responses due to chronic hypoxic incubation (Crossley and Altimiras, 2005). In the present study, we hypothesized that acute 10% O_2 exposure would induce a hypometabolic state in American alligator (*Alligator mississippiensis*) embryos, but that chronic hypoxic-incubated embryos would display a metabolic rate similar to normoxic-incubated embryos when both groups were measured in normoxia, 21% O_2. Our findings suggest cardiovascular phenotypic changes resulting from hypoxic incubation enable embryonic alligators to sustain metabolic rate during acute hypoxic exposure.

2. Materials and methods

2.1. Embryo acquisition and incubation

American alligator (*Alligator mississippiensis*) eggs were collected in 2011 and 2015 from Rockefeller Wildlife Refuge in Grand Chenier, LA, USA and transported to the University of North Texas, Denton, TX. Egg mass was determined to the nearest mg (Mettler Toledo XS204; Columbus, OH, USA), and two eggs from each clutch were used each year to establish embryonic stage (days post-laying) for each clutch as previously described (72 day incubation period at 30 °C; (Ferguson, 1985; Crossley and Altimiras, 2005). Equal numbers of eggs from each clutch were randomly distributed to plastic containers (2.5 L Ziploc® Container, SC Johnson, Racine, WI) and buried to the egg midpoint in 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 2–3 times weekly and adding water as needed to keep the mass constant (Crossley and Altimiras, 2005; Eme and Crossley, 2015).

Incubation in experimental conditions began at ~20% of embryonic development (~14 days post laying) when containers were sealed inside large Ziploc® bags with two holes that allowed parallel inflow and outflow of gas. The hypoxic gas mixture (10% O_2) was created using compressed N_2 and room air connected to two rotameters (Sho-Rate Brooks Instruments Division, Hatfield, PA). Room air was pumped directly into the bag containing the 21% O_2 group of eggs. The 10% O_2 incubation condition has previously been shown to have no impact on total incubation time, which was verified with a subset of embryonic alligators in each year of this study (Crossley and Altimiras, 2005; Owerkowicz et al., 2009). Gas mixtures passed through a H_2O-bubbler to ensure adequate water saturation of ≥80–95% relative humidity. Gas composition was monitored continuously with an oxygen analyzer (S-3AI, Applied Electrochemistry, IL, USA) connected to a PowerLab 16/35® data recording system connected to a computer running LabChart Pro® software (v 7.2 ADInstruments, CO, USA), and data recorded at 10 Hz (Eme et al., 2011b).

2.2. Study I

In 2011, two eggs from each of two clutches and four eggs from each of two clutches were divided equally and then randomly assigned to a 21% O_2 normoxic ('N'; $N = 6$) or a 10% O_2 hypoxic ('H'; $N = 6$) incubated group. At approximately 30% of total incubation and onwards, periodic oxygen consumption rate (VO_2) measurements at 21% O_2 (normoxia) began and continued at 10% of incubation intervals in the study (i.e., every 6–8 days of the ~72 day incubation) from 30 to 90% of incubation. VO_2 was measured for $N = 6$ embryos from each group, N or H, with VO_2 for each embryo measured once at 30% and 90% of incubation. Two VO_2 measures were conducted within 2 days on each embryo at 40%, 50%, 60%, 70%, 80% of total incubation and the average of the 2 measures used as the VO_2 value for the embryo.

2.3. Study II

In 2015, eight eggs from each of four clutches, six eggs from each of two clutches, and two eggs from each of two clutches were divided equally and then randomly assigned to the N ($N = 24$) or H ($N = 24$) incubated group. At approximately 30% of total incubation and onwards, periodic VO_2 and carbon dioxide production rate (VCO_2) measurements at 21% O_2 (normoxia) or 10% O_2 (hypoxia) began, and respiratory quotient (RQ) was calculated; these measurements and calculation continued at 20% of incubation intervals in the study (i.e., every 13–15 days) from 30 to 90% of incubation. VO_2 and VCO_2 were measured sequentially once in each of two conditions for $N = 6$ embryos from each group, N and H.

After measurements were completed, embryos were euthanized by placing the egg in a sealed container (250 ml) with isoflurane saturated cotton gauze for 10 min. Egg, embryo, and yolk masses as well as embryo stage (age) were determined following euthanasia for all embryos in Study I and II (Ferguson, 1985).

2.4. Metabolic measurements

For both Studies, closed system respirometry was used as previously described (Eme et al., 2011b). Eggs were removed from incubation and weighed to the nearest mg (Mettler Toledo XS204). Egg volume was determined using the egg dimensions in accordance with prior work (Maritz and Douglas, 1994) and verified for each egg by volume displacement in a 30 °C constant temperature room. For Study I only, VO_2 was measured with CO_2 scrubbed (soda lime) prior to gas sample measurements as previously reported (Warburton et al., 1995; Eme et al., 2011b). For Study II both VO_2 and VCO_2 were determined using previously published methods (Warburton et al., 1995; Eme et al., 2011b). For measurements at 21% O_2, eggs were individually sealed for 30–120 min (1–2% fall in oxygen concentration) within an airtight, 250 ml mason jar/metabolic chamber fitted with two 20-gauge needles attached to the lids connected to 3-way stopcocks. Each chamber was flushed with 30 °C room air at 100 ml min^{-1} for 5 min prior to sealing the chamber.

For Study II, metabolic measurements at 21% O_2 were made first, and measurements at 10% O_2 made 24 h subsequent. For 10% O_2 measurements, metabolic chambers were flushed with 10% O_2 for 5 min at 100 ml min^{-1}. For all metabolic measurements, a 30-ml air sample