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Effects of regional hypoxia and incubation temperature on growth, differentiation, heart mass, and oxygen consumption in embryos of the leopard gecko (*Eublepharis macularius*)

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ABSTRACT

Oviparous reptile embryos must tolerate fluctuations in oxygen availability and incubation temperature during development. In this study, regional hypoxia was simulated by painting eggs of *Eublepharis macularius* with melted paraffin wax to decrease the available surface area for gas exchange by approximately 80%. Experimental and control eggs were incubated at either 28 or 34 °C and embryo mass, stage, heart mass, relative heart mass, and oxygen consumption ($\dot{V}O_2$) were measured at 15 and 30 days of incubation. Embryo mass from the regional hypoxia treatment was reduced by about 50% at day 15 and by about 30% at day 30 of incubation, independent of incubation temperature compared to controls. Embryo stage from the regional hypoxia treatment was reduced by about 2 stages at day 15 independent of incubation temperature but there was no effect of hypoxia treatment at day 30. Absolute heart mass was reduced by about 60% in regional hypoxia embryos sampled at day 15 while relative heart mass was increased by about 30% in regional hypoxic embryos at day 30 compared to controls, suggesting that heart mass is conserved at the expense of somatic growth. Embryo $\dot{V}O_2$ was affected by incubation temperature at both 15 and 30 days of incubation but not by regional hypoxia treatment. These results indicate that embryos of *E. macularius* possess plasticity in their capacity to respond to reduction in oxygen availability during incubation, and are able to survive and continue developing when gas exchange surface area is severely limited.

1. Introduction

Embryos of oviparous reptiles experience fluctuations in environmental conditions within the nest during incubation (e.g. Ackerman, 1980; Christian and Lawrence, 1991; Booth and Thompson, 1991; Shine and Harlow, 1996; Booth, 2006; Du and Shine, 2010). Selection of favorable nesting sites by gravid females presumably helps increase embryo survival by reducing the probability of egg predation (Bowman and Harris, 1980) as well as by reducing exposure to extremes of temperature, hydric, and oxygen conditions (Christian and Lawrence, 1991; Warner and Andrews, 2002; Liang et al., 2015). Nonetheless, even after accounting for maternal nest site selection, embryos must be able to tolerate fluctuations in physical environment within the nest that may occur on a daily or seasonal basis (reviewed in Du and Shine, 2015).

Appropriate range of incubation temperature and adequate oxygen availability are two environmental factors necessary for successful embryonic development resulting in viable neonates (Packard et al., 1977; Andrews et al., 1997; Andrews, 2002). Temperature influences embryonic development through direct effects on rates of biochemical processes such as genetic transcription/translation and via indirect effects on metabolic oxygen consumption (Rombough, 2011; Zuo et al., 2012). Both rate of growth in embryo mass as well as rate of anatomical development increase as a function of temperature with a concomitant increase in embryo oxygen consumption to supply tissue metabolic demand (Vleck and Hoyt, 1991; Thompson and Stewart, 1997). The rate of anatomical development is greatest during approximately the first half of embryonic development with formation of the neural tube, organs, jaws, and limbs (Dufaure and Hubert, 1961). During this period, increase in embryo mass is relatively small and corresponding overall O_2 consumption is relatively low. The latter half of development, however, is characterized by a rapid increase in somatic mass and overall O_2 consumption, but a relatively slow rate of change in embryo stage. Because embryonic development is an aerobic process, a

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temperature-dependent increase in developmental rate is predicted to continue until tissue oxygen demand exceeds available oxygen supply (Frazier et al., 2001; Andrews, 2002; Liang et al., 2015; Smith et al., 2015). At this inflection point, increased temperature no longer enhances development but rather results in retarded development or embryo death due to the mismatch between embryo oxygen demand and availability.

Respiratory gas exchange by embryos of oviparous amniotes occurs via the vascular chorioallantoic membrane (CAM) (Wangensteen et al., 1970; Blackburn, 1993; Birchard and Reiber, 1993; Maina, 2017). In most lizards, the chorioallantois forms at approximately stage 30–31 of development (Dufaure and Hubert, 1961) and assumes the primary avenue for O_2 uptake during organogenesis and onset of rapid increase in embryo mass (Thompson and Stewart, 1997). The chorioallantois increases in size during development, eventually covering the majority of the eggshell's inner surface and maximizing surface area available for O_2 uptake (Stewart and Florian, 2000). Under natural conditions, reptile embryos may experience regional hypoxia in which the gas exchange surface area is limited. Regional hypoxia can occur due to a variety of causes such as close packing of eggs against one another in a nest (Ackerman, 1977; Ackerman, 1980), contact with the body of the female as in brooding pythons (Stahlschmidt and Denardo, 2009), or due to eggshell saturation with water (Parker et al., 2004; Tang et al., 2017). Under these conditions, embryos must employ compensatory physiological responses to maintain oxygen homeostasis. For example, prolonged exposure to hypoxic conditions may result in cardiac hypertrophy, an acclimatory response presumably compensating for increased blood viscosity due to elevated serum erythrocyte concentration (Tazawa et al., 1971; Warburton et al., 1995; Crossley and Altimiras, 2005).

The aim of this study was to determine the effects of incubation temperature and reduction of respiratory membrane surface area (regional hypoxia) on growth, anatomical development, O_2 consumption ($\dot{V}O_2$), and heart mass of leopard gecko embryos (*Eublepharis macularius,* Gekkonidae) at early and late stages of development. Leopard geckos were chosen for this study because they produce parchment-shell eggs typical of most squamate reptiles, they are easy to breed in a laboratory, and females produce multiple clutches of eggs (two eggs per clutch) over an approximate five-month reproductive season. Developmental rate of *E. macularius* embryos are typical of many lepidosaurs with the duration of incubation ranging from about 45 days at 28 °C (Parker, unpublished data) to about 36 days at 33 °C (Viets et al., 1993). Available surface area for gas exchange was reduced by painting the egg surface with melted paraffin wax leaving only the initial area covered by the chorioallantoic membrane at oviposition (i.e. approximately 20% of total egg surface area) available for gas exchange. We predicted that during early development, when embryo mass is small, growth in mass, anatomical development, growth in heart mass, and $\dot{V}O_2$ are temperature dependent, but relatively insensitive to effects of regional hypoxia. Accordingly, there should be no interactive effect of regional hypoxia treatment and incubation temperature on embryo development or $\dot{V}O_2$. Long term incubation at constant temperatures of 34–36 °C likely approaches the upper thermal limit for *E. macularius* embryos based on decreasing embryonic developmental rate at these temperatures (Viets et al., 1993). Consequently, during late development, we predicted that embryo growth in mass, anatomical development, growth in heart mass, and $\dot{V}O_2$ are dependent on egg gas exchange surface area due to the overall increased somatic mass and associated energetic demands of the developing embryo. During late development, we predicted that embryos incubated at warm (34 °C) temperature under regional hypoxia conditions have retarded growth and differentiation compared to eggs incubated at cool (28 °C) incubation temperature under regional hypoxia conditions due to the mismatch between embryo O_2 demand and O_2 availability.

2. Materials and methods

2.1. Source of eggs and experimental manipulations

Eggs of *Eublepharis macularius* were obtained from February 2014 – September 2015 from a breeding colony consisting of eight female/male pairs maintained in an animal facility at Coastal Carolina University. Eggs from each clutch were collected within 24 h of oviposition and weighed (nearest 0.01 g) on an electronic balance. To assess effects of regional hypoxia on embryonic development, one egg from each clutch was painted with melted paraffin wax (Gulf Wax ™, Household Paraffin Wax) leaving only the vascularized area surrounding the embryo uncovered. The vascularized area was identified by candling eggs and drawing a circle around the perimeter of the vascularized area using a Sharpie ™ pen. Melted wax was applied to the surface of the non-vascularized portion of the egg using a paintbrush. The temperature of the wax in the paintbrush was approximately 47 °C at the time of application to the surface of the egg. Wax temperature in the paintbrush was recorded using a copper-constantan thermocouple thermometer (Model 800024C, Sper Scientific, Scottsdale, Arizona, USA). Immediately after application of melted paraffin, the egg was cooled by holding it in front of the cooling vent of a standard 0 °C commercial freezer for approximately 5 s. On average, the initial surface area of the vascularized region was approximately 2 cm^2 and the average total surface area of eggs estimated from egg mass (Paganelli et al., 1974) was 12.5 cm^2. Thus, regional hypoxia treatment reduced egg surface area by approximately 80% compared to controls. Experimental eggs were weighed after application of wax to determine the additional mass of wax added to the egg. Control eggs did not receive wax treatment but were wrapped individually with a single layer of plastic food wrap and briefly immersed (ca. 2 s) into water heated to 45–50 °C to simulate wax application. After immersion in heated water, the plastic wrap was removed and both experimental and control eggs were placed in specimen jars containing vermiculite moistened with water (0.7:1.0 g H_2O–vermiculite) corresponding to a water potential of approximately −200 kPa for incubation and sealed with plastic Saran ™ food wrap. The plastic wrap is permeable to oxygen and CO_2 but not water vapor, thus eggs were able to freely exchange respiratory gases with the surrounding atmosphere while air within the egg incubation cups was saturated with water vapor.

To evaluate effects of regional hypoxia on differentiation and growth, we imposed wax treatments at one of two time points: 1) within 48 h of oviposition, (n = 25), or 2) 15 days after oviposition (n = 20). Leopard geckos lay eggs with embryos at approximately stage 28 to 30 (Wise et al., 2009), which corresponds to the modal embryo stage at oviposition for the majority of oviparous lizard species (Andrews and Mathies, 2000). Major developmental events associated with stages 28–30 include organogenesis, initiation of limb bud development, and the beginning of the early growth phase. At 15 days of incubation (stage 34–36), growth in somatic mass increases, whereas much of the major anatomical differentiation such as formation of organs, head, jaws, and limbs, is nearing completion.

Eggs assigned to the day 15 time point were incubated at either 28 °C (n = 12) or 34 °C (n = 13) for 15 days. On day 15 of incubation, oxygen consumption was measured (described below), and eggs weighed and fixed in 10% neutral buffered formalin. Eggs assigned to the day 30 time point were incubated at 28 °C for 15 days, wax applied, and allocated to 28 °C (n = 10) or 34 °C (n = 10) temperature treatments. On day 30 of incubation, eggs from 28 and 34 °C temperature treatments were measured for oxygen consumption (described below), weighed, followed by fixation in 10% neutral buffered formalin.

2.2. Determination of embryo stage, embryo mass, and heart mass

Formalin-fixed embryos were dissected from extra-embryonic membranes and staged using the Dufaure and Hubert (1961) staging