Oxygen availability affects behavioural thermoregulation of turtle embryos

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Oxygen Availability Affects Behavioural Thermoregulation of Turtle Embryos

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Hypoxia Affects Thermal Preference

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Abstract  Extending recent findings that reptile embryos seek optimal temperatures inside eggs for thermoregulation, our study demonstrates that this thermoregulatory behaviour can be affected by the amount of oxygen available to an embryo. We exposed embryos of a freshwater turtle (Mauremys reevesii) to two heat sources (an optimal temperature of 30 °C and a high temperature of 33 °C) under three different oxygen levels – hypoxia (12% O\textsubscript{2}), normoxia (21% O\textsubscript{2}) and hyperoxia (30% O\textsubscript{2}) – and quantified the interactive effects of temperature and oxygen availability on embryonic thermoregulatory behaviour. Our results demonstrated that, in both thermal treatments, embryos exposed to hypoxia did not move as °C lose to the heat source and therefore selected lower temperatures than those exposed to normoxia or hyperoxia. Embryos may select low temperatures under hypoxic conditions to decrease oxygen consumption and therefore alleviate the negative impact of hypoxic stress.

Keywords  behavioural thermoregulation, embryonic development, heat tolerance, hypoxia, reptile
1. Introduction

Unlike endotherms that maintain high body temperature via internal thermogenesis (Angilletta et al., 2010), most ectothermic animals behaviourally thermoregulate to utilize environmental heat sources, and thereby achieve optimal body temperatures that maximize their functional performance (Angilletta et al., 2002). Behavioural thermoregulation by ectotherms is very important for their daily activity, but may be affected by a number of biotic and abiotic factors (e.g. food, competition, predation and oxygen availability) (Herczeg et al., 2008; Rusch and Angilletta, 2017; Wood and Glass, 1991). For example, many ectotherms select lower body temperatures as an adaptive response to protect vital organs under hypoxic stress (Wiggins and Frappell, 2002; Wood and Glass, 1991). Exploring the interaction of ecological factors on behavioural thermoregulation is critical for understanding how ectotherms cope with unpredictable environmental change.

Behavioural thermoregulation not only occurs at post-embryonic stages, but also occurs in embryos of oviparous amniotes (Du et al., 2011; Li et al., 2014; Ye et al., 2013b). The adaptive significance of this behaviour was under debate (Cordero et al., 2018; Du and Shine, 2022; Shine and Du, 2018; Telemeco et al., 2016). Thermoregulatory behavior is expected to be conducive for successful embryonic development (Du et al., 2011). A recent study showed that behavioral thermoregulation by turtle embryos influenced offspring sex ratio, which expands the range of nest temperatures producing an equal sex ratio (Ye et al., 2019). Nonetheless, the adaptive significance of this behavior warrants further studies.

In the Chinese three-keeled pond turtle (Mauremys reevesii), when eggs were exposed to lateral heating, embryos moved towards a heat source at an optimal developmental temperature of 30 °C, but moved away from a heat source at a stressful temperature of 33 °C (Zhao et al., 2013b). Given that the thermal preference of adult ectotherms can be lowered on exposure to hypoxia (Wood and Glass, 1991), we hypothesize that reptilian embryonic thermoregulatory behaviour may also be affected by oxygen availability. Further, we predict that embryos will select lower body temperatures when exposed to hypoxia than when exposed to normoxia and hyperoxia. To test this hypothesis, we conducted a manipulation experiment with a two-factor design [three oxygen levels (hypoxia, normoxia, and hyperoxia) × two thermal environments (optimal and high temperatures)] to identify the effects of
oxygen availability and temperature on the thermoregulatory behaviour of freshwater turtle embryos.

2. Material and Methods

2.1. Study species and egg collection  The Chinese three-keeled pond turtle is a freshwater turtle distributed in southern and central China as well as southeastern Asia. Females lay eggs at the bank of rivers or ponds with vegetation cover from May to August. Mean nest temperatures of this species at semi-natural breeding sites range from 25.7 °C to 30.1 °C depending on the season (Ye et al., 2019). In 2016, we collected a total of 112 fertilized *M. reevesii* eggs from a private turtle farm in Zhejiang, China. These eggs were weighed (mean ± SD, egg mass = 7.357 ± 0.932 g), measured (mean ± SD, egg length = 32.88 ± 2.15 mm), and then incubated in boxes (220 × 100 × 80 mm) with moist vermiculite (−220 kPa, 1 g water/1 g vermiculite, Zhao et al., 2013a) at a constant room temperature of 28 °C in normoxic conditions (21% O₂).

2.2. Experimental design and thermoregulation behaviour  We commenced the following experiments when embryos had reached developmental stage 16−17 (Greenbaum, 2002). First, we located the initial position of an embryo in each egg by candling and then marked the position on the eggshells (Zhao et al., 2013b). The eggs were then incubated individually in small plastic boxes filled with moist vermiculite (−220 kPa). We randomly assigned these boxes to the six treatments with three oxygen levels [hypoxia (12% O₂), normoxia (21% O₂) or hyperoxia (30% O₂)] and two lateral heating treatments (30 °C and 33 °C, optimal and high temperature temperatures for embryonic development, respectively). We sealed plastic boxes with eggs into a 30 L polybag, which was supplied with gas containing different levels of oxygen. The polybags were then exposed to lateral heating of 30 or 33 °C. To achieve egg-surface temperatures of 30 or 33 °C, we adjusted the distance between the electronic heating mats (45 watt, 300 × 700 mm) and the polybags accordingly (Zhao et al., 2013b). After seven days during which the distance of embryo movements is substantial and readily measured (Zhao et al., 2013b), the final position of each embryo was detected by candling. We recorded the distances between the initial and final positions of an embryo along the long axis of the egg with a vernier caliper (±0.01 mm). The distance embryos moved towards or away from a heat source was used as a measure of the behavioural thermoregulatory ability of embryos.
During incubation, oxygen concentrations in the gas polybags were achieved by mixing oxygen with nitrogen. The gas in each polybag was renewed every day to maintain the desired oxygen concentration. During gas renewal, freshwater was added to the vermiculite to maintain the water potential of moist vermiculite. The process of gas renewal and freshwater addition was completed in under 2 minutes. We sampled gas from polybags periodically before and after each renewal. The sampled gas was checked by O\(_2\) and CO\(_2\) analyzers (FC-10A and CA-10A; Model TR3, Sable Systems, Henderson, NV, USA) with O\(_2\) and CO\(_2\) sensors (UI-2; Model TR3, Sable Systems, Henderson, NV, USA). During the experiment, the O\(_2\) level of the gas in each polybag was kept within 2\% of the three required treatment values (12\%, 21\% or 30\% O\(_2\)), and the CO\(_2\) level kept below 0.2\%.

2.3. Data analysis  We used two-way ANOVAs to analyze the effect of lateral heating treatment and oxygen concentration on the distance of embryonic movements towards or away from the heat source. Given the significant interaction between heating treatment and oxygen concentration, ANOVAs were used to analyze the effect of oxygen concentration on embryonic movement in each thermal treatment, and post-hoc tests (Tukey's test) were used to identify differences among oxygen treatments.

3. Results

The distances moved by embryonic Chinese three-keeled pond turtles were significantly affected by temperature (\(F_{1,106} = 28.996, P < 0.0001\)), oxygen concentration (\(F_{2,106} = 25.008, P < 0.0001\)), and the interaction between temperature and oxygen availability (\(F_{2,106} = 3.084, P < 0.05\)). In general, embryos from the 30 °C treatment moved greater distances towards the heat source than those from the 33 °C treatment, which did not move as far or as close to the heat source (Figure 1). The distance that embryos moved towards the heat source decreased as oxygen concentration declined in both lateral heating treatments of 30 °C (\(F_{1,56} = 10.061, P < 0.001\)) and 33 °C (\(F_{1,50} = 21.814, P < 0.0001\)). Specifically, in the 30 °C treatment embryos exposed to normoxia and hyperoxia moved towards the heat source, but those exposed to hypoxia did not; in the 33 °C treatment embryos exposed to hyperoxia moved towards the heat source, but those exposed to hypoxia and normoxia moved away from the heat source (Figure 1). Therefore, the distance embryos moved towards a heat source decreased as oxygen concentration declined in both lateral heating treatments of 30 °C and 33 °C.
4. Discussion

Our study concurs with the conclusion from a previous study (Zhao et al., 2013b) that turtle embryos at normal oxygen levels move towards a heat source when exposed to an optimal temperature of 30 °C, but move away from a heat source when exposed to a high temperature of 33 °C. More importantly, consistent with our prediction, we demonstrated that oxygen availability during development significantly affects behavioural thermoregulation by turtle embryos; embryos exposed to hypoxia did not move as close to a heat source and, therefore, selected lower temperatures than embryos exposed to normoxia or hyperoxia in both thermal treatments.

This phenomenon of hypoxia-induced hypothermia has also been seen in post-embryonic stages of ectothermic animals (Petersen et al., 2003; Wiggins and Frappell, 2002; Wood and Glass, 1991). In adult reptiles, downregulation of their thermoregulatory setpoint is triggered by the release of adenosine due to a decrease in arterial oxygen (Petersen et al., 2003). The benefits of the regulated decrease in body temperature under hypoxia include improving oxygen loading in the lungs, reducing energetically costly cardiac hyperactivity, and decreasing oxygen consumption given the thermal sensitivity of metabolism (Sun et al., 2015; Wang et al., 1998). Therefore, embryonic selection of lower temperatures under hypoxia is a beneficial strategy that may alleviate the negative impact of hypoxic stress on development.

Reptile eggs in a nest likely experience hypoxia when unpredictable climate events such as excess rainfall and flooding occur, and when metabolism of microbes and eggs deplete the oxygen within the nest chamber (Ackerman and Lott, 2004; Seymour et al., 1986). When facing hypoxia, reptile embryos may increase chorioallantoic membrane vascular density, shunt blood flow away from non-exchanging areas, increase cardiac output and carrying capacity of blood O₂, or enlarge the opaque white patch (Corona and Warburton, 2000; Tang et al., 2018). In addition to these physiological adjustments in response to hypoxia, our study demonstrates the adaptive response of embryonic thermoregulatory behaviour to hypoxia in turtles, expanding our knowledge of the capabilities that embryos possess to cope with unpredictable environments.

Hypoxia decreases the heat tolerance of ectotherms, because decreasing oxygen supply cannot meet the increasing demands of oxygen consumption when environmental temperatures increase, as suggested by the hypothesis of oxygen- and
Recent studies have demonstrated that oxygen supply significantly affects heat tolerance of reptile embryos, including turtles and lizards (Liang et al., 2015; Smith et al., 2015). Our results suggest that hypoxia not only decreases heat tolerance, but also the thermal preference of embryos. However, further studies are needed to fully understand the interactive effects of oxygen availability and temperature on the behaviour and physiology of ectothermic embryos.

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Figure 1 Effects of oxygen concentration and thermal treatment on the behavioural thermoregulation of embryos in the Chinese three-keeled pond turtle (*Mauremys reevesii*). Hypoxia, normoxia, and hyperoxia are 12%, 21% and 30% oxygen concentrations, respectively. The numbers above the error bars are sample sizes. Means with different alphabets above the error bars are statistically different among different oxygen concentrations in each temperature treatment. The dots below and above the boxes are outliers.
Figure 1  Effects of oxygen concentration and thermal treatment on the behavioural thermoregulation of embryos in the Chinese three-keeled pond turtle (Mauremys reevesii). Hypoxia, normoxia, and hyperoxia are 12%, 21% and 30% oxygen concentrations, respectively. The numbers above the error bars are sample sizes. Means with different alphabets above the error bars are statistically different among different oxygen concentrations in each temperature treatment. The dots below and above the boxes are outliers.