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SHORT COMMUNICATION

Effects of low-oxygen conditions on embryo growth in the painted turtle, *Chrysemys picta*

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Abstract

Low-oxygen conditions (hypoxia; <21% O_2) are considered unfavorable for growth; yet, embryos of many vertebrate taxa develop successfully in hypoxic subterranean environments. Although enhanced tolerance to hypoxia has been demonstrated in adult reptiles, such as in the painted turtle (*Chrysemys picta*), its effects on sensitive embryo life stages warrant attention. We tested the hypothesis that short-term hypoxia negatively affects growth during day 40 of development in *C. picta*, when O_2 demands are highest in embryos. A brief, but severe, hypoxic event (5% O_2 for 0.5 h) moderately affected embryo growth, causing a 13% reduction in mass (relative to a normoxic control). The same condition had no effect during day 27; instead, a nearly anoxic event (1% O_2 for 72 h) caused a 5% mass reduction. All embryos survived the egg incubation period. Our study supports the assumption that reptilian embryos are resilient to intermittently low O_2 in subterranean nests. Further work is needed to ascertain responses to suboptimal O_2 levels while undergoing dynamic changes in developmental physiology.

Key words: embryo metabolism, embryo respiration, growth rate, nest environment, oxygen consumption rate

INTRODUCTION

Organisms are perhaps most vulnerable to environmental unpredictability during embryonic life stages. In addition to moisture and temperature, fluctuations in oxygen (O_2) may influence normal development of vital organs, survival and growth of vertebrate embryos (Kam & Lillywhite 1994; Fisher & Burggren 2007; Dunwoodie 2009; Lungman and Piña 2013; Liang *et al.* 2015; Smith *et al.* 2015). In oviparous tetrapods, embryo growth may be disrupted when gaseous O_2 in the egg incubation environment decreases below normal atmospheric conditions (<21%; hypoxia hereafter) (Stock & Metcalfe 1987; Kam 1993a; Warburton *et al.* 1995; Dzialowski *et al.* 2002; Parker *et al.* 2004; Chan & Burggren 2005; Eme *et al.* 2013; Tate *et al.* 2015). Still, many tetrapods (e.g. megapode birds and non-avian rep-

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tiles) develop in subterranean nests that periodically experience hypoxia (Seymour & Ackerman 1980; Packard & Packard 1988; Booth & Thompson 1991; Booth 1998).

Available data from natural nests of non-avian reptiles (reptiles hereafter) indicate that O_2 concentration may fluctuate between normoxic and hypoxic levels (10–21%) (Thompson 1981; Booth & Thompson 1991; Booth 1998). Hypoxic conditions generally depend on biotic factors (e.g. embryo and microbial respiration, and clutch size), as well as abiotic factors, such as water saturation of soil (Packard & Packard 1988; Ackerman & Lott 2004; reviewed in Chen *et al.* 2010). The extent to which soil is saturated by water may determine the duration and severity of hypoxic conditions. For instance, severe hypoxic events are brief in natural turtle nests, lasting several hours after heavy rainfall (Booth 1998). Moreover, physical characteristics of soil, independent of water saturation, may contribute to hypoxia (Packard & Packard 1988; Ackerman & Lott 2004).

Physiological responses of reptilian embryos in subterranean nests vary according to developmental stage of cardiovascular and gas-exchange organs, which may exhibit increased efficiency in O_2 transport during hypoxia (Kam 1993a; Corona & Warburton 2000; Crossley & Altimiras 2005). This form of developmental plasticity may enable normal O_2 consumption during recurring hypoxic events (Kam 1993b). Furthermore, physiological sensitivity to hypoxia depends on the volume of O_2 required to fuel rapid embryo growth during the last third of the egg incubation period, when the rate of embryo growth increases rapidly in vertebrates (Thompson 1989; Zonneveld & Kooijman 1993; Tullis & Peterson 2000). Consequently, growth rate reduction is an evolutionarily conserved physiological response to hypoxia experienced late in embryonic development of vertebrates (Kajimura *et al.* 2005).

Late stages of embryonic development in turtles are particularly sensitive to hypoxia because high metabolic activity related to growth requires acceleration of the O_2 consumption rate (Ackerman 1981; Packard & Packard 1988; Tate *et al.* 2015). Hypoxia late in development may cause a reduction in the growth rate, as well as mortality, due to decreased O_2 consumption in late-term embryos (Ackerman 1981; Kam 1993a, 1994; Tucker *et al.* 1997; Eme *et al.* 2013; Tate *et al.* 2015). By contrast, enhanced tolerance to exceptional hypoxic conditions experienced seasonally in the adult life stage has been demonstrated in turtles that overwinter at high latitudes, such as the painted turtle (Emydidae: *Chrysemys picta*) (Bickler & Buck 2007; Jackson *et al.* 2007). Like other vertebrates, *C. picta* reaches peaks in rates of growth and O_2 consumption during the last third of development (Lynn & Von Brand 1945; Peterson & Kruegl 2005). During this time, decreased O_2 consumption due to hypoxia likely causes reduction in embryo growth, as demonstrated in other emydid turtles (e.g. *Pseudemys nelsoni*; Kam 1993a, 1994).

The objective of the present study was to examine whether brief hypoxic events, as experienced in nature, have effects on embryo growth during sensitive windows of developmental physiology. Thus, we tested the hypothesis that embryo growth during day 40 of development would be more sensitive to short-term hypoxia than during day 27. Day 40 coincides with the expected peak in O_2 consumption rate in *C. picta* (Peterson & Kruegl 2005).

MATERIALS AND METHODS

Egg collection and incubation

Clutches of eggs of *C. picta bellii* (western painted turtle; Fig. 1) were collected from Beem Lake, near Hyannis, Nebraska, USA (described in Rowe 1994), on 7–8 June 2012 and 12–13 June 2013. Within 48 h of oviposition, eggs were transported to Iowa State University, weighed, and half-buried in a moist vermiculite substrate (water potential = −150 kPa) in plastic boxes with lids. Eggs were incubated in a single environmental chamber set to a constant 27 °C, which yields a 100% male sex ratio in *C. picta* (Etchberger *et al.* 1992). Egg boxes were rotated weekly to control for potential thermal gradients and vermiculite was rehydrated if needed. All boxes were treated identically, with the exception of hypoxic conditions described below.

Experimental design

Our experimental design resembles recently published methodologies for inducing hypoxia during incubation of turtle eggs (Eme *et al.* 2013; Liang *et al.* 2015; Tate *et al.* 2015). Clutches of eggs collected in 2012 (N = 15; mean clutch size: 5.9 ± 0.63 SE) were randomly assigned to three treatments in Experiment 1: normoxia control (21% O_2), hypoxia (5% O_2 for 0.5 h) during stage 20 (St. 20_hypoxia; day 27), and hypoxia (5% O_2 for 0.5 h) during early stage 22 (St. 22'_hypoxia; day 40). In Experiment 2, clutches of eggs collected in 2013 (N = 6; mean clutch size: 7.5 ± 1.7 SE) were randomly assigned to two treatments: normoxia control (21% O_2), and hypoxia (1% O_2 for 72 h) during stage 20 (St.

Figure 1 Hatchling *Chrysemys picta bellii* after emergence from its subterranean nest.

20_hypoxia; day 27). Experiment 2 tested the prediction that *C. picta* embryos are more tolerant to hypoxia during stage 20 of Cordero and Janzen (2014).

Rationale for treatments

Fractional O_2 concentrations of 1%, 5%, and 21% corresponded to partial pressures (PO_2) 7.36, 36.8 and 154.6 Torr. In natural nests of freshwater turtles, PO_2 of <100 Torr (<13% O_2) lasting up to several days has been recorded following heavy rainfall (e.g. *Chelodina expansa*; Booth 1998). In addition, flooding of nests likely causes extreme hypoxia (e.g. 1%–5% O_2) (Kam 1994; Tucker *et al.* 1997). Thus, our experimental treatments corresponded to field conditions. Similar laboratory manipulations elicited physiological responses in close taxonomic relatives of *C. picta*: continuous hypoxia at 5% O_2 induced mortality in the closely related *Trachemys scripta* (Etchberger *et al.* 1991); brief hypoxia at 10% O_2 (0.5 h) reduced heart rate (i.e. O_2 transport) in *Emys orbicularis* (Nechaeva 2011); and hypoxia due to experimentally-induced flooding of nests lasting 72 h reduced rates of growth and O_2 consumption in embryos of *P. nelsoni* (Kam 1994).

Oxygen consumption rate

Oxygen consumption rate ($\dot{V}O_2$) was measured using stop-flow respirometry. Eggs at St. 20 (day 27) and St. 22' (day 40) were placed individually in 500-mL glass jars with vermiculite substrate (−150 kPa) held at a constant 27 °C. Each jar was flushed and then sealed for 90 min. Air flow (500 mL/min) was then restored, water was scrubbed from air using Drierite desiccant, and O_2 and carbon dioxide (CO_2) were measured using CA-10 and FC-10 analyzers, respectively (Sable Systems International). Oxygen consumption rate was calculated by integrating the change in instantaneous level over the period the jars were sealed, after correcting for barometric pressure and CO_2, using ExpeData software (Sable Systems). This subset of eggs ($N = 6$) was excluded from other analyses.

Oxygen manipulation

A manually operated gas-mixing flow meter was used to gradually introduce nitrogen (N_2) gas to original egg boxes, now sealed with parafilm, until reaching targeted O_2 concentrations, which were monitored in real time using fiber optic sensor technology (Fibox 3, PreSens Precision Sensing, Germany). This setup included a temperature-compensated oxygen sensor (Loligo Systems, Denmark) with O_2 and temperature recorded by OxyView PST3-V5.32 software (PreSens Precision Sensing). Gases introduced to egg-containing boxes traveled through heat-exchanging coils that maintained temperature at 27 °C. Eggs in experimental groups began incubation in 21% O_2 and were returned to that con-

dition following hypoxic treatments. All egg boxes were weighed before and after experimental manipulation to ensure that water potential of egg substrate remained at −150 kPa. Fractional O_2 concentrations were corrected for barometric pressure, temperature and water vapor. During normal incubation, we did not observe random deviation from normal atmospheric O_2 (21%) in egg-containing boxes.

Data collection

Sampling in this study adhered to guidelines for animal care and use approved by the ISU Institutional Care and Use Committee (protocol # 2-11-7091-J). Euthanasia of embryos occurred *in ovo* via overnight fixation in 10% buffered formalin following standard protocol for preservation of reptilian embryos (Foster 2012). Eggs were fixed on day 52 (late stage 22), before yolk sac internalization (Mahmoud *et al.* 1973). To quantify stage-specific embryo mass, eggs incubated in normoxia were subsampled on days 27 and 40. Fixed eggs were washed in distilled water to avoid tissue shrinkage (Stowell 1941). Embryos were then excised and residual yolk sacs (except in Experiment 2) and other extraembryonic tissues were discarded. Carapace length was measured to the nearest 0.1 mm along the anterior–posterior axis using digital calipers, following standard embryological methods for *C. picta* (Cordero & Janzen 2014). The anterior–posterior axis is mostly calcified and relatively undistorted by the flexible ellipse-shaped egg of emydids (Ewert 1985); thus, it can be measured reliably at the end of egg incubation (e.g. Mahmoud *et al.* 1973; Packard *et al.* 1983; Cordero & Janzen 2014).

Yolk-free dry embryo mass, a more inclusive indicator of growth in embryonic turtles (Ewert 1985), was used to infer differences in energy allocated to production of tissue during development (Ar *et al.* 2004). Yolk-free embryos and separated yolk sacs (Experiment 2) were dried to a constant mass in an oven set to 60 °C for 72 h. Yolk-free dry embryo mass and dry residual yolk mass (Experiment 2) were recorded to the nearest 0.001 g using an electronic balance. Embryo survival was inferred by examining external egg conditions (Ewert 1985), and egg pipping (≥1 egg/group) was observed to determine completion of egg incubation because it is less variable than hatching time in *C. picta* (Gutzke *et al.* 1984). Egg pipping also indicates termination of the extended growth phase (stage 22; Cordero & Janzen 2014).

Statistical analyses

Maternal egg-energy investment (i.e. initial egg mass) can vary substantially among years in our study population (Rowe 1994). Therefore, data collected in 2012 (Experiment 1) and 2013 (Experiment 2) were analyzed separately. The effect of hypoxia on yolk-free dry embryo mass was tested using mixed-effect analysis of covariance (ANCOVA), with clutch of origin as a random factor and the following covariates: carapace length (Experiments 1 and 2), initial egg mass (Experiments 1 and 2) and dry residual yolk mass (Experiment 2 only). Carapace length and initial egg mass are strong predictors of wet hatchling mass in our study population (Rowe 1995). Initial egg mass controls for energetic maternal investment and carapace length for size.

Interaction terms, as well as initial egg mass and dry residual yolk mass, were excluded because they were not significant in preliminary models (all P values > 0.18). Significant terms ($P < 0.0001$) for carapace length and initial egg mass (Experiment 1 only) were retained in the final models. In all experimental groups, yolk-free dry embryo mass was normally distributed (Shapiro–Wilk's test, all P values > 0.11). Tukey's honest significant difference tests were used to evaluate mean differences among treatments. Covariate-adjusted means and their standard errors are reported. Analyses were conducted in the *nlme* package of the R programming language (R Core Team 2014).

RESULTS

In Experiment 1, brief hypoxia (0.5 h at 5% O_2) had an effect on yolk-free dry embryo mass measured at the end of egg incubation (ANCOVA, $F_{2,55} = 4.7034$, $P = 0.013$, $N = 74$), which was lower in the St. 22'_hypoxia (day 40; $N = 24$) treatment than in the St. 20_hypoxia (day 27; $N = 26$) and control groups (Fig. 2a). This resulted in a 13% mass reduction in St. 22'_hypoxia embryos relative to the normoxic control ($N = 24$). The yolk-free dry embryo mass in the St. 20_hypoxia treatment did not differ from that in the control group (Fig. 2a). In Experiment 2, severe hypoxia (72 h at 1% O_2) beginning on day 27 (St. 20_hypoxia; $N = 14$) affected yolk-free dry embryo mass measured at the end of egg incubation (ANCOVA, $F_{1,19} = 8.080$, $P = 0.0104$; $N = 27$) (Fig. 2b). This resulted in a 5% mass reduction relative to the normoxic control ($N = 13$).

Yolk-free dry mass increased from 0.035 g ± 0.001 (day 27, St. 20; $N = 5$) to 0.177 g ± 0.003 (day 40, St. 22'; $N = 5$) under normoxic incubation. In addition, the

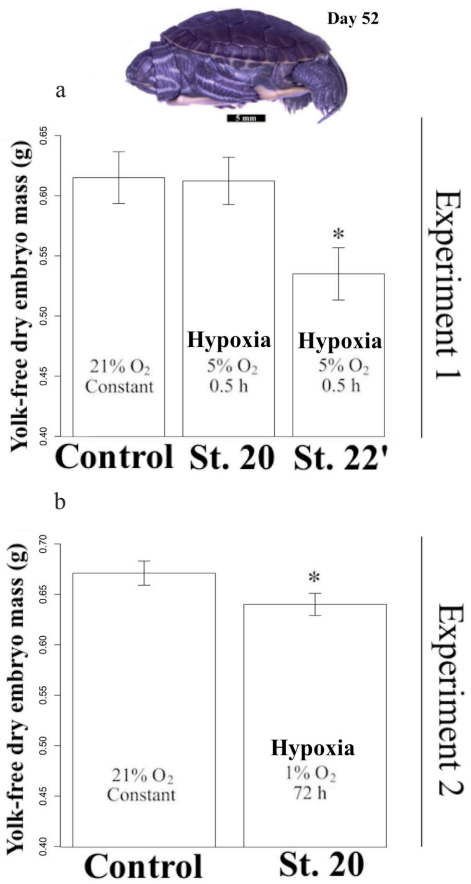

Figure 2 Means (± SE) of yolk-free dry embryo mass (adjusted for carapace length and initial egg mass) in *Chrysemys picta bellii* differed only in control (21% O_2) versus the St. 22'_hypoxia (5% O_2; day 40) groups (a; Experiment 1). In Experiment 2 (b), covariate-adjusted means of the control (21% O_2) and St. 20_hypoxia (1% O_2; day 27) groups differed. Yolk-free dry embryo mass and carapace length were recorded at the end of egg incubation (around day 52 at 27 °C); *Statistically significant mean difference (P< 0.05).

rate of O_2 consumption increased by more than twofold: $\dot{V}O_2$: 0.352 mL/h ± 0.028 (day 27, St. 20; N = 4) versus $\dot{V}O_2$: 0.859 mL/h ± 0.032 (day 40, St. 22'; N = 6). All embryos survived to the end of egg incubation: around day 52 at 27 °C. Means for initial egg mass, carapace length and yolk-free dry embryo mass are reported in Table 1.

DISCUSSION

We report evidence of growth reduction in *C. picta* caused by short-term hypoxia in the egg incubation environment. Embryos briefly exposed to hypoxia on day 40, when high levels of O_2 are required to fuel rapid growth, exhibited a decrease in yolk-free dry mass relative to embryos in normoxia. As expected, the same hypoxic condition, but earlier in development, had little or no detectable effect on embryo mass. Notably, there was only a slight reduction in embryo growth after exposure to nearly anoxic conditions over 72 h. In *C. picta picta* from New Jersey, O_2 consumption rate increased nearly twofold from day 27 to day 40 (Peterson & Kruegl 2005). During this time frame, we demonstrated that the O_2 consumption rate increased twofold in *C. picta bellii*. Furthermore, estimates of embryo size and mass, as well as length of incubation, were comparable to in *C. picta bellii* populations near our study site in Nebraska (Packard *et al.* 1983; Rowe 1995; reviewed in Cordero & Janzen 2014).

Overall, our findings are biologically informative in revealing that: (i) brief, although severe, hypoxia is sufficient to reduce the growth rate during a narrow window in development of *C. picta*; (ii) reduction of the growth rate is dependent on the timing and magnitude

Table 1 Means of yolk-free dry embryo mass and carapace length (measured at the end of egg incubation; approximately day 52 at 27 °C), as well as initial egg mass in *Chrysemys picta bellii*

Group	Initial egg mass (g ± SE)	Yolk-free dry embryo mass (g ± SE)	Carapace length (mm ± SE)
Experiment 1[+]			
Control	5.48 ± 0.154	0.615 ± 0.034	21.6 ± 0.30
St. 20_hypoxia	5.56 ± 0.172	0.612 ± 0.020	21.9 ± 0.29
St. 22'_hypoxia	5.53 ± 0.121	0.535 ± 0.022	22.4 ± 0.24
Experiment 2[+]			
Control	6.91 ± 0.116	0.671 ± 0.012	22.9 ± 0.24
St. 20_hypoxia	7.01 ± 0.134	0.640 ± 0.011	23.3 ± 0.20

+ = In Experiment 1: hypoxia was set to 5% O_2 for 0.5 h on day 27 (stage [St.] 20) and day 40 (St. 22'); in Experiment 2, hypoxia was set to 1% O_2 for 72 h on day 27 (St. 20). Control: constant 21% O_2.

of hypoxia; and (iii) embryos of *C. picta* survive and possibly resume normal growth after experiencing exceptionally low O_2. No prior experiment had subjected turtle embryos to such an extreme condition without placing eggs in substrate oversaturated with water (e.g. Kam 1994; Tucker *et al.* 1997).

Resilience to hypoxia

Survival of reptile embryos in subterranean nests is likely enabled by physiological adjustments that enhance O_2 transport during hypoxic events, including hypervascularization and hypertrophy of gas-exchange organs (Kam 1993a; Corona & Warburton 2000; Crossley & Altimiras 2005; Nechaeva 2011). Although *C. picta* is remarkable in its capacity to withstand extreme hypoxic conditions experienced in the adult life stage (Bickler & Buck 2007; Jackson *et al.* 2007), our results do not suggest that tolerance to hypoxia during embryo life stages is exceptional relative to other turtles. When considering the duration of hypoxic conditions, the observed reduction in embryo growth was comparable to similar experiments on other turtles, which also did not detect effects on survival and length of incubation period (Kam 1993b; Tucker *et al*. 1997; Eme *et al.* 2013; Tate *et al*. 2015). However, survival and incubation period in turtles may be affected if temperature is suboptimal during hypoxia (Liang *et al.* 2015). Overall, our results corroborate that embryos in earlier stages of development are resilient to hypoxia.

Narrow window of sensitivity to hypoxia?

Recent work has identified sensitive windows of physiological sensitivity to prolonged hypoxia (10% O_2 at up to 636 h) that corresponded with alteration of cardiovascular function and decreased embryo growth in laboratory-incubated snapping turtles (Chelydridae: *Chelydra serpentina*; Tate *et al.* 2015). Both hypoxic time intervals in our study were brief (0.5 and 72 h) and within the critical windows described for *C. serpentina*. However, the severity of hypoxia in our experiment was greater (1% or 5% O_2) and concurrent with the expected peaks in rates of O_2 consumption and growth in *C. picta* (Lynn & Von Brand 1945; Packard *et al.* 1983; Peterson & Kruegl 2005). Our results suggest that critical windows of sensitivity to hypoxia are narrower, at least with respect to overall growth of embryos. Crucially, hypoxic events are brief in natural nests of freshwater turtles (Booth 1998).

Ecological and evolutionary implications

Reduction of embryo growth due to hypoxia is likely an evolutionarily conserved physiological response that increases the likelihood of survival when O_2 available for respiration is limited (Kajimura *et al.* 2005). Accordingly, we showed that *C. picta* embryos with reduced mass caused by hypoxia survived the egg incubation period, as in other turtles (Etchberger *et al*. 1991; Kam 1993b; Eme *et al*. 2013; Tate *et al*. 2015). However, environmental fluctuation in subterranean nests of reptiles might affect fitness-related hatchling traits (Deeming 2004). In *C. picta*, hatchling size is considered a fitness-relevant trait because it is associated with increased likelihood of survival (Paitz *et al.* 2007). Therefore, whether physiological responses to hypoxia influence hatchling phenotypes, and potentially fitness, in *C. picta* needs to be addressed.

Eggs in natural nests of freshwater turtles likely experience brief hypoxia following heavy rainfall (Booth 1998). Although O_2 data indicating hypoxia in natural nests of *C. picta* are not available, flooding of nests causes high embryo mortality (Christens & Bider 1987; Janzen 1994). This was supported by experiments that induced hypoxia by simulating flood events (Kam 1994; Tucker *et al.* 1997). Flooding also affects temperature and moisture, both of which exert combinatorial effects on the metabolism of reptilian embryos (Kam & Lillywhite 1994; Lungman & Piña 2013; Liang *et al.* 2015; Smith *et al.* 2015).

Future studies should aim to examine multiple traits (e.g. heart rate and growth, and hatchling phenotypes), as well as interactions of moisture, temperature and O_2 to comprehensively characterize how physiological systems respond to fluctuating O_2 availability in subterranean nests. Still, our study is an important contribution towards illuminating effects of O_2 limitation during particularly sensitive periods of embryonic development in vertebrate animals.

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