

Oxygen-dependent heat tolerance and developmental plasticity in turtle embryos

Liang Liang · Bao-Jun Sun · Liang Ma · Wei-Guo Du

Received: 1 August 2014 / Revised: 28 October 2014 / Accepted: 4 November 2014 / Published online: 19 December 2014
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Abstract Oxygen and temperature have previously been treated as different environmental stresses and studied separately in most cases. Although the oxygen–temperature interaction may provide new insight into proximate and evolutionary constraints on embryonic development and offspring fitness, it has rarely been studied in oviparous amniotes. We used a two-factor experiment [three oxygen concentrations (12, 22 and 30 %) × two temperatures (26.5 and 34 °C)] to identify the effect of the oxygen–temperature interaction on embryonic development and hatchling traits in the Chinese soft-shelled turtle, *Pelodiscus sinensis*. When eggs were incubated at the critically high temperature, hatching success of turtle eggs was enhanced by hyperoxia but reduced by hypoxia; this result was not observed in eggs incubated at the benign temperature. Hypoxia retarded embryonic development, and reduced body size, locomotor performance and survival rate of hatchlings at the critically high temperature. However, the effects of hypoxia were greatly reduced at the benign temperature. Our study demonstrates that oxygen and temperature interact to affect not only the heat tolerance and developmental rate of embryos but also the fitness-related traits of hatchlings, suggesting that interactions among environmental factors impose significant ecological constraints on embryonic development in oviparous amniotes.

Keywords Critical thermal maximum · Embryonic development · Hyperoxia · Hypoxia · Oxygen limitation · Reptile · Thermal tolerance

Introduction

Levels of atmospheric oxygen and temperature have fluctuated considerably (from 12 % to over 30 %) over the past 550 million years (Bergman et al. 2004; Berner 2006; Huber et al. 2000). The interaction between oxygen and temperature not only affects the behaviour and physiology of organisms, but may also have influenced the course of biological evolution (Berner et al. 2007; Huey and Ward 2005; Pörtner 2010). For example, temperature increase causes oxygen limitation for marine fish by reducing aquatic dissolved oxygen and by enhancing the animals' oxygen consumption (Pörtner and Knust 2007); low oxygen plus warm temperatures have been hypothesised to have led to a catastrophic extinction at the end of the Permian period (Huey and Ward 2005). Despite the importance of the oxygen–temperature interaction for organismal physiology and survival, these two environmental factors generally have been treated as different environmental stresses and hence, studied separately (Pörtner 2010; Willmer et al. 2004). However, the oxygen–temperature interaction, which has received increasing scientific attention recently (Huey and Ward 2005; Pörtner 2010), may shed light on both phenotypic plasticity and evolutionary adaptation of organisms in response to environmental changes.

Unlike viviparous embryos that develop inside the maternal body with stable temperature and oxygen supply, the embryo within an oviparous amniote egg develops in an external nest and may be exposed to a wide and unpredictable range of oxygen and temperature availability. For example, oxygen

Communicated by I. D. Hume.

L. Liang · B.-J. Sun · L. Ma · W.-G. Du (✉)
Key Laboratory of Animal Ecology and Conservation
Biology, Institute of Zoology, Chinese Academy of Sciences,
Beijing 100101, People's Republic of China
e-mail: duweiguo@ioz.ac.cn

declines significantly after rain or if metabolic activities of microbes and sibling eggs deplete the oxygen within the nest chamber (Ackerman and Lott 2004; Booth 2000; Seymour et al. 1986); nest temperatures fluctuate dramatically in reptiles (most notably in shallow nests) or when brooding parent birds leave the nest to forage (Jia et al. 2010; Shine et al. 2003). In addition, oviparous amniotes produce eggs with parchment or rigid shells that not only provide a protective environment for the embryo, but may also restrict oxygen influx (Packard and DeMarco 1991). High temperature and hypoxia are two crucial environmental stresses that oviparous amniote embryos may face during development. Previous studies have demonstrated that oxygen and temperature can significantly affect embryonic development and the resultant hatchlings in oviparous amniotes (see reviews by Deeming 2004; Deeming and Ferguson 1991; DuRant et al. 2013; Nechaeva 2011), but the vast majority of these studies focus on single-factor effects rather than among-factor interactions. For example, amniote embryos at benign temperatures would develop into larger hatchlings that performed better than those at high or low temperatures, and extremely high or low temperatures could be harmful or even lethal for amniote embryos (Booth et al. 2000; Du and Ji 2003; Ji and Du 2001; Whitehead et al. 1992). Hypoxia may reduce metabolic and growth rates of embryos, and induce cardiac hypertrophy and facultative increase of heart rate, vascular density on the chorioallantoic membrane and haematocrit in oviparous amniotes (Corona and Warburton 2000; Crossley and Altimiras 2005; Du et al. 2010; Kam 1993; Seymour et al. 1986; Snyder et al. 1982; Warburton et al. 1995). By contrast, the effect of hyperoxia on embryonic development receives much less attention, but may be important for understanding how atmospheric oxygen level affects the physiology of both extinct and contemporary vertebrates (Owerkowicz et al. 2009).

Identifying the oxygen–temperature interaction may provide new insights into the proximate and evolutionary constraints on embryonic development and offspring fitness in oviparous amniotes. First, if the interaction of temperature and oxygen affects thermal tolerance, the survival of embryos under thermal stress would depend on oxygen availability in nests. Second, even within the viable oxygen and temperature ranges, temperature–oxygen interaction is likely to significantly modify hatchling phenotypes and to severely affect offspring fitness, because both high temperature and hypoxia have negative effects on embryonic development and hatchling traits (Deeming 2004; Nechaeva 2011).

The Chinese soft-shelled turtle (*Pelodiscus sinensis*) lives in rivers, lakes and ponds, and lays clutches of small rigid-shelled eggs from May to August in eastern China (Zhang et al. 1998). The rigid-shelled eggs have low gas permeability (Deeming and Thompson 1991; Overgaard et al. 2007), making this an ideal species to identify oxygen-dependent thermal tolerance and development in amniote embryos. We used a

two-factor (oxygen concentration \times temperature) experimental design to test for the effects of oxygen and temperature on the thermal tolerance and development of embryos and the phenotypic traits of hatchlings in the Chinese soft-shelled turtle. We predicted that (1) hatching success at the critically high temperature would be higher for turtle embryos exposed to hyperoxia than for those exposed to hypoxia; (2) the effects of hypoxia would be more severe at the critically high temperature than at the benign temperature.

Materials and methods

Experimental design

We used a two-factor experimental design [three oxygen concentrations (12, 22 and 30 %) \times two temperatures (26.5 and 34 °C)] to identify the effects of the oxygen–temperature interaction on embryonic development and hatchling traits. The two temperatures, 26.5 and 34 °C, are the benign and critically high temperatures for embryonic development of *P. sinensis*, respectively (Du and Ji 2003). Our pilot study indicated that, when incubated in an incubator with natural air circulation with an oxygen concentration of 20.1 %, *P. sinensis* eggs from a captive population at Hangzhou had high hatching success (97 %, or 31/32) at 26.5 °C, but much lower hatching success (50 %, or 23/46) at 34 °C. This result verified that 26.5 °C and 34 °C are the benign and critically high temperatures, respectively, for embryonic development in our study population.

Egg collection and incubation

In May, 2013, we collected 25 clutches containing 286 freshly laid and fertilised *P. sinensis* eggs from a private farm in Hangzhou city of Zhejiang Province, China. The eggs were weighed (average egg mass 5.47 ± 0.34 g), incubated in plastic boxes (220 \times 100 \times 80 mm) containing moist vermiculite (-220 kPa), and assigned to one of the six treatments in the two-factor experiment, using a split-clutch design. Each box was sealed in a 30-L polybag and supplied with hyperoxic or hypoxic gas. Oxygen concentrations were achieved by mixing compressed oxygen with nitrogen, using air cylinders. Each polybag was filled with the mixed gas until slightly inflated, and was checked twice per day to ensure that eggs were under slight positive pressure from the mixed gas supply. The gas in the polybag was renewed each day, and the gas renewal of each polybag was completed within 3 min. Meanwhile, the boxes were weighed and water added to compensate the water loss due to evaporation and absorption by the eggs and thereby maintain a relatively constant water potential of the incubation substrate. Gas from polybags was sampled periodically

before and after each renewal and checked with O₂ and CO₂ sensors (UI-2; Model TR3, Sable Systems, Henderson, NV, USA), connected to O₂ and CO₂ analysers (FC-10A and CA-10A; Model TR3, Sable Systems, Henderson, NV, USA). Throughout the experiment, the O₂ level of the gas in each polybag was maintained within 2 % of 12, 22 or 30 %, with a CO₂ level less than 0.2 %.

Incubation period, hatching success, and hatchling phenotypes

Once the first hatchling appeared, we monitored the boxes once a day for newly emerging hatchlings. The number of days elapsed between the beginning of incubation and the emergence of the hatchlings was recorded as the incubation period. Hatching success was calculated as the percentage of hatched eggs relative to incubated eggs in each treatment.

After emergence, the hatchlings were weighed (± 1 mg) and maintained in a 300-mL jar until the yolk had been entirely absorbed (2 days in most cases). The turtles were then measured for carapace size (length and width, ± 1 mm), and their locomotor performance was assessed by chasing them along a 0.8-m long straight racetrack. The trials were conducted in a room with a constant temperature of 30 °C, which is optimal for locomotor performance in this species (Wu et al. 2013). The locomotor performance of each turtle was tested twice with a half-hour resting period between trials and recorded with a SONY HDR-XR150 digital video camera. The fastest speed over 20-cm intervals was collected a posteriori from the videotapes.

Following the locomotion test, the hatchling turtles were housed individually in 500-mL jars with 5-cm-deep water in a temperature-controlled room at 30 ± 1 °C and with a 12-h light:12-h dark cycle. The turtles were fed with commercial food daily, and their survival was monitored for 2 months after hatching.

Data analysis

We conducted *G* tests to determine whether the hatching success of eggs and the survival rate of hatchlings differed among oxygen treatments in each thermal treatment. Two-way mixed-model ANOVAs were used to analyse the effects of oxygen and temperature on incubation period, with clutch number as the random factor. Two-way mixed-model ANCOVAs were used to analyse the effects of oxygen and temperature on morphology and locomotor performance of hatchlings, with initial egg mass as a covariate and clutch number as the random factor. Given the significant effect of the interaction between oxygen and temperature on incubation period and hatchling traits, we further ran one-way mixed-model ANOVAs or ANCOVAs

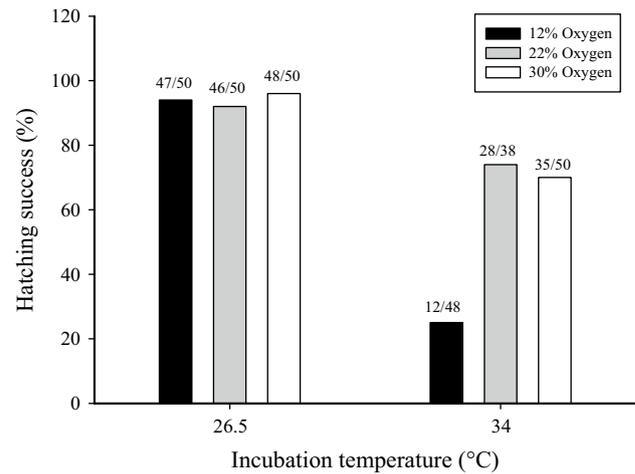


Fig. 1 Hatching success of turtle eggs (*P. sinensis*) from different oxygen and temperature treatments. Numbers above the error bar are sample sizes. Hatching success in hyperoxia (30 %) and near normoxia (22 %) were higher than those incubated in hypoxia (12 %) when incubated at a critically high temperature, but not at a benign temperature

to determine the effect of oxygen in each temperature treatment. Means are presented \pm one standard error and results were deemed to be significant if $P < 0.05$.

Results

Hatching success was affected by oxygen treatment at the critically high temperature of 34 °C, with lower hatching success for eggs exposed to 12 % oxygen than for eggs exposed to 22 and 30 % oxygen ($G = 28.2$, $df = 3$, $P < 0.001$). In contrast, hatchling success was not affected by oxygen treatment at the benign temperature of 26.5 °C ($G = 0.72$, $df = 3$, $P > 0.05$) (Fig. 1).

Incubation period was significantly affected by incubation temperature ($F_{1,89} = 9,338.9$, $P < 0.0001$), oxygen concentration ($F_{2,89} = 26.9$, $P < 0.0001$), and the interaction between them ($F_{2,89} = 13.4$, $P < 0.0001$). Eggs took longer to hatch when incubated at 26.5 °C than 34 °C (Fig. 2). At both temperatures, oxygen concentration significantly affected incubation period (26.5 °C: $F_{2,66} = 9.2$, $P < 0.001$; 34 °C: $F_{2,23} = 40.0$, $P < 0.0001$). Eggs exposed to 12 % oxygen took longer to hatch than those exposed to 22 and 30 % oxygen, and the among-treatment difference was greater at 34 °C (3 days) than at 26.5 °C (1 day) (Fig. 2).

Oxygen concentration, incubation temperature and the interaction between them significantly affected hatchling locomotor performance and all morphological traits measured (Table 1). When incubated at 34 °C, hatchlings from the 22 and 30 % oxygen treatments were considerably larger and heavier than their siblings from the 12 % oxygen treatment

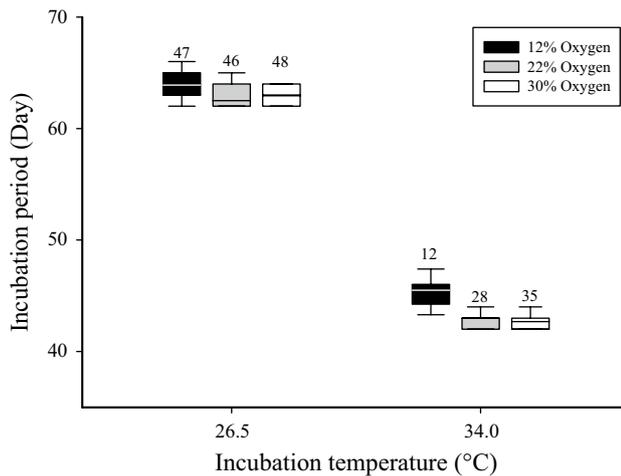


Fig. 2 Incubation period of turtle eggs (*P. sinensis*) from different oxygen and temperature treatments. Data are expressed as mean \pm SE. Numbers above the error bar are sample sizes. Incubation period was longer when eggs were incubated at a benign temperature than at a critically high temperature, and in hypoxia (12 %) than near normoxia (22 %) or hyperoxia (30 %)

(body mass- $F_{2,22} = 11.5$, $P < 0.001$; carapace length-34 °C: $F_{2,22} = 29.1$, $P < 0.0001$; carapace width- $F_{2,22} = 27.6$, $P < 0.0001$) (Fig. 3). However, this effect was greatly reduced at 26.5 °C (body mass- $F_{2,65} = 8.9$, $P < 0.001$; carapace length- $F_{2,65} = 13.7$, $P < 0.0001$; carapace width- $F_{2,65} = 7.1$, $P < 0.01$) (Fig. 3). Hatchlings from the 22 and 30 % oxygen treatments had larger heads than their siblings from the 12 % oxygen treatment when incubated at 34 °C (head length- $F_{2,22} = 16.5$, $P < 0.0001$; head width- $F_{2,22} = 10.9$, $P < 0.001$). In contrast, this difference was only seen in head length ($F_{2,65} = 3.8$, $P < 0.05$), but not in head width ($F_{2,65} = 0.25$, $P = 0.78$) in hatchlings from eggs incubated at 26.5 °C (Fig. 4). Hatchlings from the 22 % oxygen treatment crawled faster than their siblings from the 12 and 30 % oxygen treatments when incubated at 34 °C ($F_{2,22} = 13.6$, $P < 0.0001$), but not at 26.5 °C ($F_{2,65} = 0.09$, $P = 0.92$) (Fig. 5).

When incubated at 34 °C, survival rate within 2 months was highest in hatchlings from the 22 % oxygen treatment

(85.7 %), lowest in hatchlings from the 12 % oxygen treatment (25.0 %); hatchlings from the 30 % oxygen treatment were intermediate (65.7 %) ($G = 14.0$, $df = 3$, $P < 0.01$). In contrast, all hatchlings from eggs incubated at 26.5 °C survived to 2 months after hatching, regardless of oxygen treatment.

Discussion

Hatching success of our turtle eggs was enhanced by hyperoxia and reduced by hypoxia when the eggs were incubated at a critically high temperature, but not when the eggs were incubated at a benign temperature. When incubated in air, *P. sinensis* eggs from the population used in this study achieve 50 % hatching success at 34 °C; eggs from another population averaged only 32 % hatching success (Choo and Chou 1987). Compared with those incubated in air, hatching success of *P. sinensis* eggs incubated in hyperoxia increased, but those incubated in hypoxia decreased (Fig. 1). Our results are consistent with previous findings from studies on embryonic and larval stages of crustaceans (Storch et al. 2009; Woods and Hill 2004), as well as studies on the adult stage of marine invertebrates, some terrestrial arthropods and fish (Klok et al. 2004; Pörtner 2010; Pörtner and Knust 2007; Stevens et al. 2010). Conversely, oxygen limitation of heat tolerance is unlikely in simple organisms such as single-celled eukaryotes and prokaryotes (Pörtner 2001, 2002) and many insects (Klok et al. 2004; Stevens et al. 2010). Simple organisms diffuse sufficient oxygen directly into tissues and cells and thus have very high heat tolerance. In contrast, higher organisms like metazoans have complex circulatory and gas exchange systems and thus much lower heat tolerance (Pörtner 2001, 2002). Analogously, oxygen limitation of heat tolerance is less prominent in most insects which have a primarily diffusion-driven tracheal system that delivers oxygen to tissues, compared to terrestrial isopods in which oxygen is delivered to tissues primarily by a circulatory system (Klok et al. 2004; Stevens et al. 2010).

Table 1 Results of ANCOVA for oxygen concentration and temperature effects on morphology and locomotor performance in hatchling *P. sinensis*

	Oxygen	Temperature	Interaction
Hatchling mass	$F_{2,88} = 19.8$, $P < 0.0001$	$F_{1,88} = 12.2$, $P < 0.01$	$F_{2,88} = 7.5$, $P < 0.01$
Carapace length	$F_{2,88} = 34.0$, $P < 0.0001$	$F_{1,88} = 14.7$, $P < 0.001$	$F_{2,88} = 24.8$, $P < 0.0001$
Carapace width	$F_{2,88} = 41.0$, $P < 0.0001$	$F_{1,88} = 163.7$, $P < 0.0001$	$F_{2,88} = 22.9$, $P < 0.0001$
Head length	$F_{2,88} = 23.1$, $P < 0.0001$	$F_{1,88} = 63.9$, $P < 0.0001$	$F_{2,88} = 15.2$, $P < 0.0001$
Head width	$F_{2,88} = 13.6$, $P < 0.0001$	$F_{1,88} = 61.4$, $P < 0.0001$	$F_{2,88} = 19.9$, $P < 0.0001$
Crawl speed	$F_{2,88} = 7.5$, $P < 0.01$	$F_{1,88} = 31.6$, $P < 0.0001$	$F_{2,88} = 6.2$, $P < 0.01$

ANCOVAs were conducted on hatchling size, head size and crawl speed, with initial egg mass as a covariate on hatchling size, and body mass on head size and crawl speed

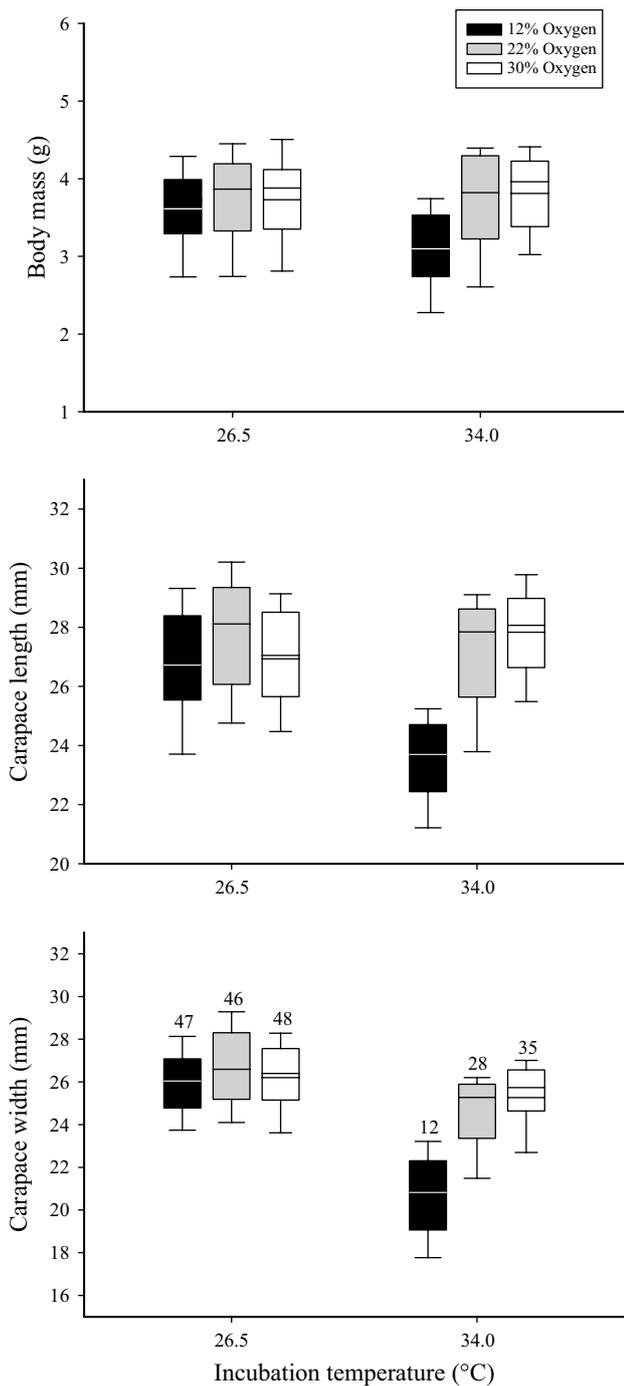


Fig. 3 Effects of oxygen concentration and temperature on body size of hatchling turtles (*P. sinensis*). Data are expressed as mean \pm SE. Numbers above the error bar are sample sizes in the bottom graph and are applicable to the upper graphs of this figure. Hatchlings incubated in hyperoxia (30 %) and near normoxia (22 %) were heavier and larger than those incubated in hypoxia (12 %) when incubated at a critically high temperature, but not at a benign temperature

Interestingly, heat tolerance (as shown by hatching success at 34 °C) of *P. sinensis* embryos increased as oxygen concentration increased from 12 to 22 %, but did not

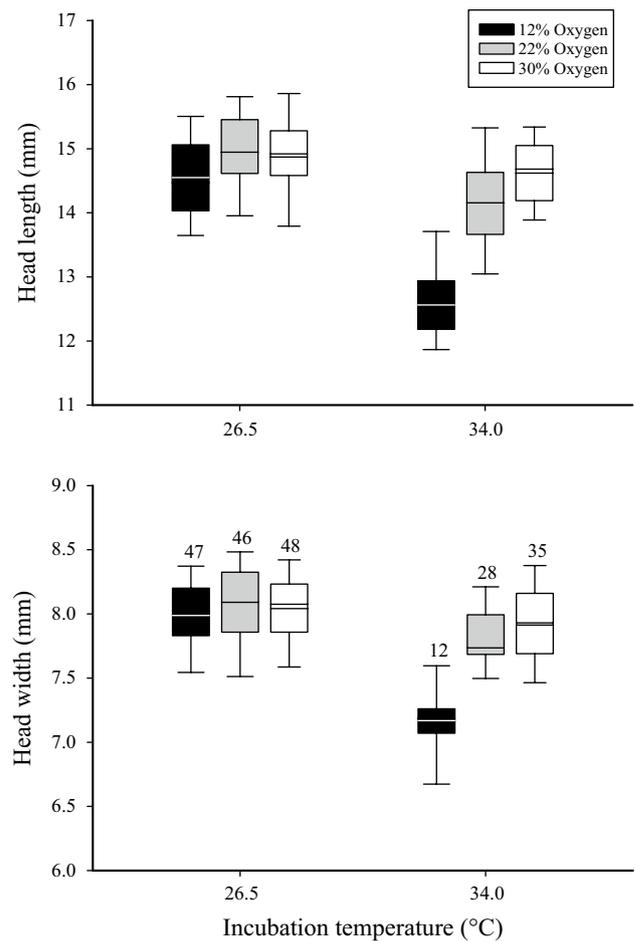


Fig. 4 Effects of oxygen concentration and temperature on the head size of hatchling turtles (*P. sinensis*). Data are expressed as mean \pm SE. Numbers above the error bar are sample sizes in the bottom graph and are applicable to the upper graphs of this figure. Hatchlings incubated in hyperoxia (30 %) and near normoxia (22 %) had larger heads than those incubated in hypoxia (12 %) when incubated at a critically high temperature, but not at a benign temperature

increase further when oxygen concentration increased to 30 % (Fig. 1). This suggests that the oxygen available for mitochondrial aerobic respiration in cells not only depends on the partial pressure of oxygen in the air (which may affect the partial pressure gradient between an organism and its environment and in turn the diffusion efficiency of oxygen), but may also be limited by the efficiency of oxygen transport to tissues and cells. The delivery of oxygen from air to tissues and cells in amniotic embryos involves three processes: diffusive gas exchange through the shell and shell membranes to capillaries in the chorioallantois, convective transport via the circulatory system, and diffusive exchange between the tissue capillaries and the cells (Andrews 2004; Burggren and Pinder 1991). In addition to oxygen transport, mitochondrial function may play an important role in oxygen limitation of thermal tolerance. For example, warm

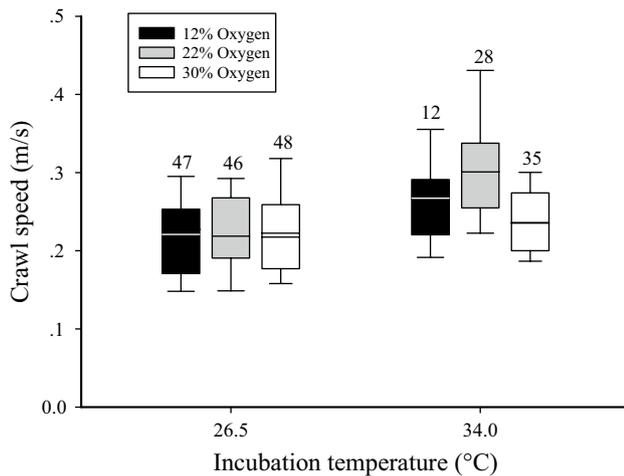


Fig. 5 Effects of oxygen concentration and temperature on locomotor performance of hatchling turtles (*P. sinensis*). Hatchlings incubated in near normoxia (22 %) and hypoxia (12 %) crawled faster than those incubated in hyperoxia (30 %) when incubated at a critically high temperature, but not at a benign temperature

acclimation leads to a reduction in mitochondrial density, which may reduce oxygen demand and shift the upper critical temperature to a higher value (Pörtner 2001), whereas cold acclimation increases mitochondrial density or mitochondrial aerobic capacity (Guderley 2004). Consequently, temperature-induced alterations of mitochondrial densities and functions may shift oxygen-limited thermal tolerance windows (Pörtner 2001). Such mechanisms have not yet been explored in terrestrial vertebrates, and provide ample opportunities for future studies.

Rarely has the effect of the interaction between oxygen and temperature on embryonic development and offspring fitness been studied in oviparous amniotes. In the domestic chicken egg, there was no evidence of an interactive effect of oxygen and temperature on embryonic development (Lourens et al. 2007), but our study demonstrated that oxygen and temperature interact to affect the developmental rate of embryos and the fitness-related phenotypes of hatchlings in a turtle, including body size, crawl speed and survival. At a critically high temperature, hatchlings incubated in hypoxia (12 %) were smaller than their siblings incubated in near normoxia (22 %) and hyperoxia (30 %) (Fig. 3), consistent with previous results from other species from major clade of vertebrates including fish, amphibia, reptiles, birds and mammals (Mills and Barnhart 1999; Nechaeva 2011; Owerkowicz et al. 2009; Sundt-Hansen et al. 2007). The small body size of hypoxia hatchlings is due to the constraint of oxygen supply, which reduces the yolk catabolism and somatic growth of embryos during development (Crossley and Altimiras 2005; Kam 1993; Owerkowicz et al. 2009). Interestingly, the body mass (carcass plus residual yolk) of hypoxia hatchlings was also smaller than

that of their siblings. A potential explanation to the smaller absolute mass of the hatchlings is that hypoxia may hamper water metabolism during development, with less water absorbed and incorporated by the embryos. Further studies on water contents of residual yolk and hatchlings are needed to clarify this hypothesis. Unexpectedly, however, at the critically high temperature, the “best” hatchlings (in terms of locomotor performance and survival rate) were those incubated in near normoxia rather than those incubated in hyperoxia (Fig. 5). This may be because the hatchlings were raised under normoxia, which may not be able to meet the demand of hypermetabolism induced by hyperoxia during embryonic development (Nechaeva 2011; Sbrong and Dzi-alowski 2007). In contrast, when eggs were incubated at 26.5 °C, the effects of hypoxia on hatchling body size were small (Fig. 3), and its effect on crawl speed and hatchling survival rate was nearly imperceptible (Fig. 5). Why were the effects of hypoxia less severe at the benign temperature than at the critically high temperature? First, oxygen limitation at the benign temperature may not be as severe as at the critically high temperature, because oxygen demand for aerobic metabolism decreases significantly at the benign temperature, whereas the capacity for oxygen supply is not reduced (Pörtner 2010). Second, embryos likely have the capability to maintain relatively constant internal oxygen levels in spite of decreased external oxygen supply, through compensatory developmental changes in their oxygen transport cascade. Some reptile and bird embryos increase the efficiency of oxygen delivery to cope with hypoxic conditions via facultative shifts in the morphology and physiology of their cardiac and vascular systems, such as heart rate, vascular density and blood parameters (Corona and Warburton 2000; Crossley and Altimiras 2005; Du et al. 2010; Miller et al. 2002; Nechaeva 2011).

Acknowledgments The authors thank T. T. Wang, Y. Z. Ye, B. F. Wu and T. Li for their help in the laboratory. Research was performed under approvals from the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001). Funding This work was supported by grants from “Hundred Talents Program” of the Chinese Academy of Sciences; and Zhejiang Provincial Natural Science Foundation (Grant number: LZ13C030002).

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