

## Research



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## Physiology

# Effects of hypoxia on the thermal physiology of a high-elevation lizard: implications for upslope-shifting species

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Montane reptiles are predicted to move to higher elevations in response to climate warming. However, whether upwards-shifting reptiles will be physiologically constrained by hypoxia at higher elevations remains unknown. We investigated the effects of hypoxic conditions on preferred body temperatures ( $T_{pref}$ ) and thermal tolerance capacity of a montane lizard (*Phrynocephalus vlangalii*) from two populations on the Qinghai-Tibet Plateau. Lizards from 2600 m a.s.l. were exposed to O<sub>2</sub> levels mimicking those at 2600 m (control) and 3600 m (hypoxia treatment). Lizards from 3600 m a.s.l. were exposed to O<sub>2</sub> levels mimicking those at 3600 m (control) and 4600 m (hypoxia treatment). The  $T_{pref}$  did not differ between the control and hypoxia treatments in lizards from 2600 m. However, lizards from 3600 m selected lower body temperatures when exposed to the hypoxia treatment mimicking the O<sub>2</sub> level at 4600 m. Additionally, the hypoxia treatment induced lower critical thermal minimum ( $CT_{min}$ ) in lizards from both populations, but did not affect the critical thermal maximum ( $CT_{max}$ ) in either population. Our results imply that upwards-shifting reptiles may be constrained by hypoxia if a decrease in  $T_{pref}$  reduces thermally dependent fitness traits, despite no observed effect on their heat tolerance.

## 1. Introduction

Global warming has proven to be a major threat to biodiversity worldwide [1]. Species were predicted, and then observed, to shift their distributions upwards in response to global warming [2]. In mountainous regions, upslope-shifting is a feasible scenario for terrestrial animals exposed to a warming environment [3,4], but moving to higher elevations could be constrained by the hypoxic effects of lower oxygen supplies on behavioural and physiological phenotypes, especially in species with high metabolic thermal sensitivity [5,6]. Recently, experimental studies revealed potential physiological constraints on upward-shifting animals [7–10]. Hypoxia has been shown to affect the thermoregulatory behaviour of ectotherms, with ectothermic animals selecting lower body temperatures under severe hypoxic conditions [7]. Hypoxia-induced changes in preferred body temperatures ( $T_{pref}$ ) may reduce the fitness of ectotherms that are highly dependent on environmental temperatures [11,12]. Hypoxia may also decrease the thermal tolerance of ectotherms as predicted by the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, which was originally proposed for aquatic ectotherms [13]. Oxygen-dependent thermal tolerance is expected to constrain the physiological niche of species, which determines potential threats faced by ectotherms in the context of global

warming [14,15]. However, whether OCLTT is applicable to terrestrial animals remains controversial [7,16].

Here, we selected a high-elevation dwelling lizard (Qinghai toad-headed lizard, *Phrynocephalus vlangualii*) as our study species to evaluate how the upward-shifting of montane ectotherms might be constrained by hypoxic conditions, because this species may experience more severe hypoxic stress than low-elevation dwelling lizards when moving upwards. Given that the annual mean air temperature of Qinghai–Tibet Plateau is predicted to increase 6.4°C by the end of the twenty-first century (according to the RCP8.5) [17], we proposed an experimental scenario whereby species shifted their elevational range upward by 1000 m to compensate for a warming scenario of 6°C. Accordingly, we compared  $T_{pref}$  and thermal tolerance capacity of lizards from two populations (2600 m [control] versus 3600 m [hypoxia treatment], and 3600 m [control] versus 4600 m [hypoxia treatment]), exposed to local  $O_2$  levels and hypoxic conditions with lower  $O_2$  levels found at higher elevations. Based on the current knowledge of potential hypoxic effects on animals, we predicted that (i) lizards exposed to lower  $O_2$  levels would select lower body temperatures compared to their counterparts exposed to local (higher)  $O_2$  levels in the high-elevation population rather than the low-elevation population, because previous studies suggested that thermal preferences were insensitive to hypoxia until extreme reduction of  $O_2$  level [5,7,16]; (ii) lizards exposed to lower  $O_2$  levels would have a lower  $CT_{max}$  but a stable  $CT_{min}$  and therefore a narrower thermal tolerance window, because previous studies suggested that hypoxia lowered  $CT_{max}$  but did not affect  $CT_{min}$  in ectothermic animals [16,18,19].

## 2. Material and methods

### (a) Study species and animal husbandry

The Qinghai toad-headed lizard (*P. vlangualii*) is a viviparous lizard widely distributed over the Qinghai–Tibet Plateau, from 2000 m to 4500 m a.s.l. In July 2019, we collected adult *P. vlangualii* from two populations at two elevations (2604 m and 3597 m a.s.l.) in the Kazak Autonomous County of Aksay, Gansu Province, China. Within 2 days of collection, we transported the lizards to our laboratory in Beijing (53 m a.s.l.) and randomly assigned individuals to experimental treatments (2600 m population: Hypoxia  $n = 4\sigma 9\phi$ ; Control  $n = 5\sigma 8\phi$ ; 3600 m population: Hypoxia  $n = 6\sigma 11\phi$ ; Control  $n = 5\sigma 9\phi$ ). Lizards from the 2600 m population were kept at two normobaric oxygen concentrations: 15.54% (control) and 13.86% (hypoxia) (equivalent to  $PO_2$  at 2600 m and 3600 m a.s.l., respectively). Lizards from the 3600 m population were kept at 13.86%  $O_2$  (control) and 12.12% (hypoxia) (equivalent to  $PO_2$  at 3600 m and 4600 m a.s.l., respectively). Air temperature was maintained at 22°C and 16°C for the 2600 m and 3600 m populations, respectively (equivalent to the average daily ambient temperature in summer of the two populations). We housed lizards from each treatment of each population in three plastic containers (47 × 35 × 24 mm), with 3–6 individuals per container. Experimental oxygen concentrations were achieved by mixing compressed oxygen with nitrogen and maintained at a constant level by a digital  $O_2$ -controlled incubator (Huaxi Electronics, Changsha, China), with fresh air continuously flushed through the system. Air temperatures were maintained in climate-control rooms. Full-spectrum bulbs (35 W, UVA + UVB) suspended over each container created a thermal gradient from 08.00 to 20.00 h for lizard thermoregulation. The thermal gradients were measured by putting copper models inserted with iButton

temperature loggers (DS1921; Maxim Integrated Products, Ltd, USA) on the hot and cold ends of the container [20] and were 22–62°C and 16–59°C for the 2600 m and 3600 m populations, respectively. We shifted the position of containers in each group every 10 days to minimize potential effects of temperature and oxygen differences within the climate-controlled rooms and  $O_2$ -controlled incubators.

### (b) Preferred body temperatures and thermal tolerances

On the 18–19th day of the experiment, we measured lizard body temperatures hourly from 09.00 to 19.00 h, by inserting the probe of an electronic thermometer (UT325, Shenzhen Meter Instruments, Shenzhen, China, 0.1°C resolution) into lizard cloacae (approx. 5 mm) to record internal body temperature. To minimize interruptions, we measured body temperatures every 2 h from 09.00 to 19.00 h on the first day, and from 10.00 to 18.00 h on the second day. Conspecific interactions might affect body temperatures in these group-housed lizards, but the number of lizards per container did not differ between the control and hypoxia groups ( $t = 0.745$ , d.f. = 10,  $p = 0.473$ ), and average body temperatures were independent of the number of lizards in a container ( $R^2 = 0.001$ ,  $t = 0.286$ , d.f. = 55,  $p = 0.776$ ). We calculated the mean of body temperatures as the  $T_{pref}$  of lizards.

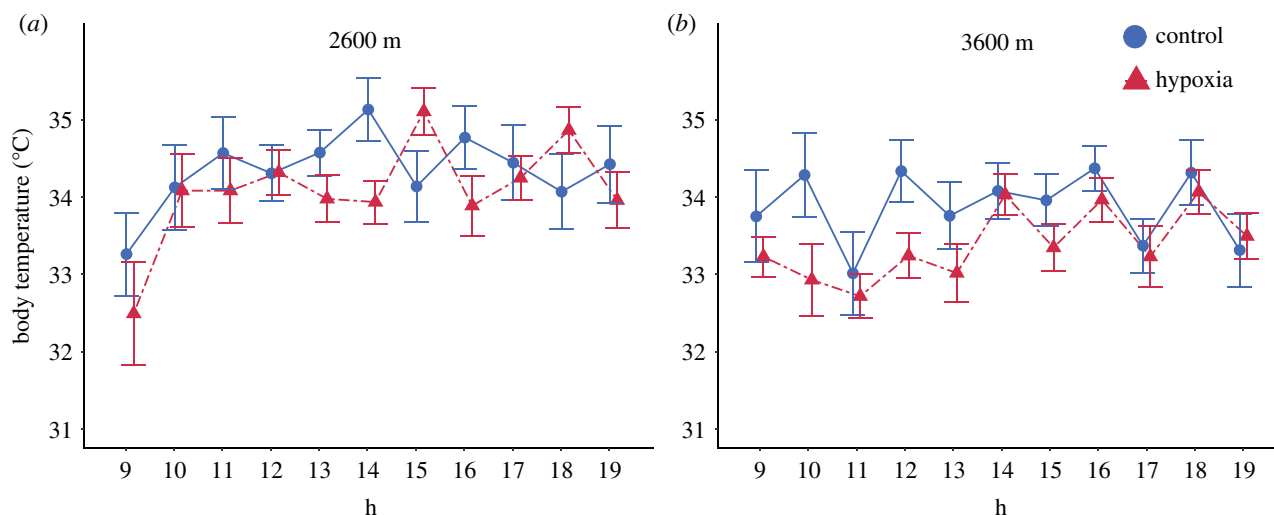
After 32 days of exposure to the experimental treatments, we measured the  $CT_{min}$  and  $CT_{max}$  of each lizard. Lizards were individually placed in a transparent cylindrical chamber (281.4 ml capacity) with oxygen at the same concentration as their respective treatments pumping in continually. To measure  $CT_{min}$ , we kept the chamber with lizards in an incubator (Sanyo, Japan) at 15°C for 1 h, and then put the chamber on ice and moved it into an incubator (Sanyo, Japan) set at –10°C, so that the lizards cooled by approximately 0.5–1°C min<sup>–1</sup> [21,22]. To measure  $CT_{max}$ , we kept the chamber with lizards in an incubator (Yuantianfukang, Nanjing, China) set at 28°C for 1 h and then increased the incubator temperature by approximately 1°C min<sup>–1</sup> [22]. During the trials, we inverted the chamber about 4–5 times per minute such that lizards were flipped onto their backs. When lizards were unable to right themselves, we immediately measured their cloacal temperatures as  $CT_{min}$  or  $CT_{max}$  using an electronic thermometer (UT325, Uni-trend technology, Guangdong, China). All lizards were then left to recover in their home containers.

### (c) Statistical analyses

We fitted Shapiro–Wilks and Bartlett tests to the data to check the normality of models' residuals and homogeneity of variances. We used linear mixed-effects models (LME; 'nlme' package) [23] to analyse the effect of hypoxia on  $T_{pref}$  in the two populations, with population and treatment as fixed factors, and individual lizard identity, container and time as random factors. LME was used to test the effect of treatments on lizard  $CT_{min}$  and  $CT_{max}$  with treatment and population as the fixed factors, and container as a random factor. We used type III sums of squares to assess the significance of main effects and used the Kenward–Roger approximation for denominator degrees of freedom [24]. We conducted all statistical analyses in R v. 4.0.3 [25].

## 3. Results

The  $T_{pref}$  of lizards did not differ between the two groups in the 2600 m population (table 1 and figure 1a), but were significantly lower in the hypoxia treatment than those from the control treatment in the 3600 m population (table 1 and figure 1b). Lizards from the 2600 m population selected higher temperatures ( $T_{pref}$ ) than those from the 3600 m population (table 1 and figure 1).



**Figure 1.** Body temperatures of the Qinghai toad-headed lizard (*P. vlangalii*) from two elevations (a) 2600 m above sea level (a.s.l.), and (b) 3600 m a.s.l. exposed to control and hypoxia treatments. Body temperatures were recorded hourly from 09.00 to 19.00 h. Error bars represent the SE. The body temperature is only comparable between control and hypoxia treatments, but not between the lizards from different elevations because they were maintained at different thermal environments.

**Table 1.** The preferred body temperature ( $T_{pref}$ ), critical thermal minimum ( $CT_{min}$ ) and critical thermal maximum ( $CT_{max}$ ) of Qinghai toad-headed lizards (*P. vlangalii*) from two elevations (2600 m and 3600 m a.s.l.), exposed to control and hypoxia treatments. The body temperature is only comparable between control and hypoxia treatments, but not between the lizards from different elevations because they were maintained at different thermal environments.

traits	elevation (m)	mean $\pm$ s.e.		statistical analysis	
		control	hypoxia	treatment	elevation
$T_{pref}$ (°C)	2600	34.28 $\pm$ 0.15	34.17 $\pm$ 0.14	$t = -0.351$ , d.f. = 24, $p = 0.728$	$t = -2.713$ , d.f. = 54, $p = 0.009$
	3600	33.87 $\pm$ 0.13	33.38 $\pm$ 0.10	$t = -2.516$ , d.f. = 29, $p = 0.018$	
$CT_{min}$ (°C)	2600	6.13 $\pm$ 0.39	4.21 $\pm$ 0.49	$t = -3.079$ , d.f. = 24, $p = 0.005$	$t = -0.500$ , d.f. = 54, $p = 0.619$
	3600	5.76 $\pm$ 0.31	4.21 $\pm$ 0.24	$t = -4.007$ , d.f. = 29, $p < 0.001$	
$CT_{max}$ (°C)	2600	47.12 $\pm$ 0.40	46.93 $\pm$ 0.22	$t = -0.402$ , d.f. = 24, $p = 0.691$	$t = 0.452$ , d.f. = 54, $p = 0.653$
	3600	46.64 $\pm$ 0.16	47.14 $\pm$ 0.23	$t = 1.693$ , d.f. = 29, $p = 0.101$	

The  $CT_{min}$  of lizards from the hypoxia treatment was lower than that of lizards from the control treatment in both populations (table 1 and electronic supplementary material, figure S1A). However, the  $CT_{max}$  of lizards did not differ between the two treatment groups from either population (table 1 and electronic supplementary material, figure S1B). Overall, the  $CT_{min}$  and  $CT_{max}$  of lizards were similar in the two populations (table 1 and electronic supplementary material, figure S1).

## 4. Discussion

Lizards from the Qinghai-Tibet Plateau selected lower body temperatures overall, but did not exhibit a lower heat tolerance following exposure to lower  $O_2$  levels, which mimicked a 1000 m increase of elevation. Moreover, the lowering of  $T_{pref}$  in response to lower  $O_2$  levels only occurred in the extremely low  $O_2$  level at 4600 m rather than the  $O_2$  level at 3600 m. Below we discuss the implications of these results in terms of potential physiological constraints on mountainous lizards shifting upslope in response to global warming.

Our hypoxia treatments (mimicking 1000 m increase of elevation) induced lizards from the 3600 m population to select lower body temperatures, giving support to our first prediction. Other ectothermic species have also been found to select lower body temperatures in response to hypoxia [5,26]. This behavioural hypothermia might act to reduce oxygen requirements and thus avoid excessive cardiac output and respiratory consumption, representing a facultative response to hypoxia [11,27]. However, hypoxia-induced lower body temperatures may also cause a decrease in functional performance and overall fitness. This is because many fitness-related traits (e.g. locomotor performance, food assimilation, anti-predator behaviour) are highly dependent on body temperatures in lizards and other ectotherms [12,28]. Nonetheless, the hypoxia-induced difference in  $T_{pref}$  is small (0.5°C, table 1), whether this small change would affect the altitudinal distribution of species is unclear. Therefore, both short- and long-term effects of hypoxia-induced low body temperatures warrant further research to evaluate the potential constraint of such conditions on upward-shifting lizards.

Interestingly, hypoxia-induced lizards to select lower body temperatures in the 3600 m population but not in the

2600 m population, possibly due to elevational variation in the severity of hypoxia i.e. the moderate hypoxia present at 2600 m has little effect on behavioural thermoregulation by lizards. For example, three species of lizards (*Iguana iguana*, *Ctenosaura pectinata* and *Varanus exanthematicus*) selected lower body temperatures under a 7–10% oxygen concentration but not when exposed to 10–21% oxygen [29]. Previous studies have demonstrated that both adults and embryos were able to cope with hypoxia via facultative physiological responses in reptiles from relatively low elevations (less than 2000 m a.s.l.) when they moved upwards to higher elevations [10,30]. Our study further suggests that populations at upper elevational range limits of high-elevation species (e.g. greater than 3000 m a.s.l.) face severe hypoxic stress, show more significant hypoxic effects on thermal preference and are thus more likely to be constrained during upwards-shifting.

Contrary to our second prediction, hypoxia had no effect on  $CT_{max}$  but lowered  $CT_{min}$  and therefore provided a greater thermal tolerance range for *P. vlangalii* in the populations we studied. The independence of  $CT_{max}$  from hypoxic conditions was also suggested by previous studies on terrestrial ectotherms [7,16]. The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis receives weak support from terrestrial air-breathing species [7,16]. Several studies have demonstrated that only severe hypoxia may induce lower  $CT_{max}$  in terrestrial animals. For example, an oxygen concentration of 7% lowered  $CT_{max}$  of the common rough woodlouse (*Porcellio scaber*) [18] and an oxygen concentration of 6% lowered  $CT_{max}$  of the western fence lizard (*Sceloporus occidentalis*) [31]. In reality, air-breathers are rarely exposed to such severe hypoxia (6–7% oxygen concentration), and  $CT_{max}$  does not vary with elevation [32]. While numerous studies have investigated the effects of hypoxia on  $CT_{max}$ , few studies have reported how hypoxia affects the  $CT_{min}$  of terrestrial ectotherms [16]. Our results showed that hypoxic conditions actually enhanced the capacity for cold tolerance in *P. vlangalii*. A potential explanation for this phenomenon is that hypoxia may upregulate transcripts of freeze-responsive genes to increase the cold tolerance of some ectothermic vertebrates [33]. By contrast, earlier studies reported that hypoxia did not affect the  $CT_{min}$  of terrestrial invertebrates [18,19]. Thus,

hypoxia at higher elevations is unlikely to constrain upwards-shifting in lizards by reducing their thermal tolerance capacity.

Our experiment sheds light on potential hypoxia-induced physiological constraints faced by upwards-shifting montane lizards under climate warming. However, it is worth noting that we kept lizards from the two populations at different temperatures mimicking their native environments. These thermal treatments enable us to determine the response of lizards to hypoxia under ecologically relevant scenarios, but may confound results on between-population difference. This caveat makes direct between-population comparisons of hypoxic effects unreliable, but does not affect our understanding of how lizards respond to hypoxia in each population, which is the focus of the present study. In addition, to achieve a sound understanding of exactly how hypoxia may constrain upwards-shifting lizards further research is needed, including long-term field experiments on reproduction and survival of lizards transferred to higher elevations. Such studies focusing on montane species (especially those populations at the upper elevational range limits) will help deepen our knowledge of how hypoxia may constrain the colonization of upwards-shifting species, and how the colonizers may adapt to novel environments both plastically and genetically.

**Ethics.** Lizard collection, handling and husbandry were approved by Animal Ethics Committees at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

**Data accessibility.** The data and R code used to generate statistics and generate figures can be found at <https://doi.org/10.5061/dryad.79cnp5htt> [34]. The electronic supplementary material, figure S1 is available as electronic supplementary material.

**Competing interests.** We declare we have no competing interests.

**Authors' contributions.** Z.W.J., L.M. and W.G.D. designed the studies. Z.W.J., L.M. and C.R.M. collected and analysed data; Z.W.J. and W.G.D. wrote the draft, and all authors contributed to revisions. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

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