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Viviparity in high-altitude *Phrynocephalus* lizards is adaptive because embryos cannot fully develop without maternal thermoregulation

Zheng Wang · Hong-Liang Lu · Li Ma · Xiang Ji

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Abstract Viviparous *Phrynocephalus* lizards (Agamidae) are mainly restricted to the Qinghai-Tibet Plateau of China. In this study, we used *Phrynocephalus vlangalii* females kept under seven thermal regimes for the whole gestation period to test the hypothesis that viviparity in high-altitude *Phrynocephalus* lizards is adaptive because embryos cannot fully develop without maternal thermoregulation. All females at 24 °C and 93 % of the females at 28 °C failed to give birth or produced stillborns, and proportionally fewer females gave birth at 29 or 35 °C than at 32 °C. Though the daily temperatures encountered were unsuitable for embryonic development, 95 % of the females in nature and 89 % of the females thermoregulating in the laboratory gave birth. There was no shift in the thermal preferences of females when they were pregnant. Although thermal conditions inside natural burrows were unsuitable for embryonic development, mass and sprint speed were both greater in neonates produced in nature. Our data show that (1) long-term exposure of *P. vlangalii* embryos to temperatures outside the range of 29–35 °C may result in the failure of development, but daily or short-term exposure may not necessarily increase embryonic mortality; (2) low gestation temperatures slow but do not arrest embryonic development, and females produce high-quality offspring in the shortest possible time by maintaining gestation temperatures close to the upper thermal limit for embryonic development; and (3) viviparity is currently adaptive at high elevations because embryos in nature cannot fully develop without relying on maternal thermoregulation. Our data validate the hypothesis tested.

Keywords Viviparity · Maternal thermoregulation · Gestation temperature · Embryonic survivorship · Offspring phenotype

Introduction

Temperature is one of the most important abiotic factors that varies both temporally and spatially, affects many biological processes and restricts species distributions (Angilletta 2009). The effects of temperature on developing embryos are more pronounced than those observed in later ontogenetic stages (Johnston et al. 1996; Lindström 1999; Deeming 2004). In reptiles, for example, the thermal conditions experienced during embryonic development affect not only hatching or birth date but also a number of offspring phenotypes, and some temperature-induced phenotypic changes may have long-term fitness consequences (Deeming 2004; Calsbeek and Sinervo 2007; Telemeco et al. 2010; Wapstra et al. 2010; Uller et al. 2011). Temperature also affects the sexual phenotype of offspring in species with temperature-dependent sex determination (Valenzuela and Lance 2004). Long-term population persistence is not possible in any area where thermal conditions are

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Z. Wang · X. Ji (✉)
Jiangsu Key Laboratory for Biodiversity and Biotechnology,
College of Life Sciences, Nanjing Normal University,
Nanjing 210046, Jiangsu, China
e-mail: xji@mail.hz.zj.cn

Z. Wang
College of Forest Resources and Environment, Nanjing Forestry
University, Nanjing 210037, Jiangsu, China

H.-L. Lu · L. Ma
Hangzhou Key Laboratory for Animal Adaptation and Evolution,
School of Life Sciences, Hangzhou Normal University,
Hangzhou 310036, Zhejiang, China

not appropriate for embryonic development, and this is especially true for species that do not migrate to reproduce (Shine 2005; Lin et al. 2007; Monasterio et al. 2011). The embryonic stage therefore provides an ideal life history phase in which to investigate a broad range of biological issues including the spatial patterns of species distribution.

Thermal adaptation leads to the evolution of morphology, physiology, behavior and life history that has made organisms better able to adapt to their environments (Angilletta 2009). One distinction that has emerged in animal life histories is that of oviparous versus viviparous reproductive modes. It is widely thought that in reptiles viviparity evolves from oviparity for thermal reasons (Andrews and Mathies 2000; Blackburn 2000; Shine 1995, 2005). All oviparous reptiles except those brooding eggs do not control thermal conditions for their eggs after oviposition (Lorioux et al. 2012), and immobile eggs cannot voluntarily regulate thermal environments for embryos (Löwenborg et al. 2010; Du et al. 2011). Thus, the distribution of oviparous reptiles is often constrained in climatically extreme regions where thermal means are too low or high for embryonic development, or thermal fluctuations exceed the range that embryos can tolerate (Shine 1995, 2005; Deeming 2004; Monasterio et al. 2011; Löwenborg et al. 2012). In viviparous taxa, however, embryos can be kept at suitable thermal levels through maternal thermoregulation. Thus, viviparity has greater selective advantages than oviparity in climatically challenging areas, although its evolution is constrained by phylogenetic, physiological and ecological factors (Shine 2005; Calderón-Espinosa et al. 2006; Bleu et al. 2012). In reptiles, viviparity has evolved only in squamates, with viviparous taxa comprising a high proportion of the squamate faunas in cold climates, although the tropics contain more viviparous taxa in absolute terms (Tinkle and Gibbons 1977). Recent studies have showed that the selective advantages of viviparity might not differ between species from climatically different areas (Webb et al. 2006; Ji et al. 2007; Li et al. 2009; Gao et al. 2010; Rodríguez-Díaz et al. 2010).

Toad-headed lizards of the reproductively bimodal genus *Phrynocephalus* are a clade of agamid lizards that are distributed in desert, arid or semiarid regions in Central and Western Asia and North–Northwestern China (Barabanov and Ananjeva 2007). These lizards have proved difficult to classify. Recent work has shed considerable light on their systematics, but also resulted in a considerable reduction in valid species. Far from <140 species, as was widely believed until the 1990s, only some 40 valid *Phrynocephalus* species are currently recognized (Barabanov and Ananjeva 2007). Of these species, six are viviparous and mainly restricted to the Qinghai-Tibet Plateau of China (Barabanov and Ananjeva 2007; Ji et al. 2009b; Noble et al. 2010). Another six, or possibly seven, *Phrynocephalus* species found in China are oviparous (Barabanov and Ananjeva 2007; Gozdzik and Fu 2009; Wang and Fu 2004). Toad-headed lizards in China are found in an altitudinal range from −40 to 6,400 m, and *Phrynocephalus erythrurus* has the highest distribution in the world (Zhao 1999). The average altitude (~3,300 m) of the viviparous taxa is higher than that (~1,200 m) of the oviparous taxa, with all viviparous species except *Phrynocephalus forsythia* appearing at altitudes higher than 2,200 m, a range within which scarcely any oviparous species can be found (Zhao 1999). Phylogenetic studies have found that the viviparous *Phrynocephalus* species form a monophyletic lineage that diverged from the oviparous taxa 9.1 ± 1.4 (SD) M years ago, with the most recent common ancestor of viviparous species dating to 4.4 ± 0.7 M years ago (Guo and Wang 2007). The uplift of the Qinghai-Tibet Plateau that led to the transition from warm and wet to cold and dry climates and the diversification of viviparous species occurred between 0.01 and 3.6 M years ago (Guo et al. 2002; Guo and Wang 2007), more recently than the time at which viviparous and oviparous lineages diverged. Thus, presumably as in viperid snakes (Lynch 2009), viviparity in toad-headed lizards is due to an innovation and adaptation to global cooling during the Cenozoic rather than to the cooling associated with the uplift of the Qinghai-Tibet Plateau. It is possible that after the uplift of the plateau, oviparous species disappeared at high elevations because external thermal environments were unsuitable for embryonic development.

Here, we use the Qinghai toad-headed lizard *Phrynocephalus vlangalii* as the model animal to determine whether thermal constraints on embryonic development provide an explanation for the occurrence of viviparous *Phrynocephalus* lizards in the Qinghai-Tibet Plateau. This viviparous lizard occurs in arid and semiarid regions in the Qinghai, Gansu, Xinjiang and Sichuan regions of China in an altitudinal range from 2,200 to 4,500 m (Zhao 1999), and females in nature give birth mostly between mid-July and mid-August (Zhang et al. 2005). All individuals of *P. vlangalii* except newborns use burrows built by themselves as refugia, and burrow fidelity is extremely high in adults (Zhang 2006). Moreover, no burrow has been found to be shared by two or more adults (Zhang 2006). We measured temperatures of natural burrows used by reproductive females, body temperatures preferred by pregnant females, non-pregnant females and adult males, the range of constant temperatures appropriate for embryonic development, and temperature-induced changes in birth date and offspring phenotypes. Data from this study allow us to test the hypothesis that viviparity is necessary for *Phrynocephalus* lizards to persist at high elevations because embryonic development is not possible without maternal thermoregulation.

Materials and methods

Thermal conditions in the field

Four burrows used by reproductive females were individually monitored to estimate the thermal environments experienced by lizards in May, July and September 2007. The four burrows were 162–367 m apart but at almost the same altitude (~3,250 m), with depths varying from 683 to 967 mm. Four U12 Hobo dataloggers (Onset, USA) with external probes were used to record surface (10 mm above the ground) and burrow (600 mm below the ground) temperatures synchronously at 1-h intervals. By combining data from this study with those collected by Zhang (2006) at depths of 100, 200, 300 and 500 mm, we established a curvilinear relationship between percentage of thermal fluctuations on the ground surface and burrow depth, testing the hypothesis of whether or not suitable environmental temperatures are available for embryonic development.

Animal collection and treatment

We collected 175 females that were ready to ovulate in early May 2007, and 40 females [thermoregulating in nature (NAT females)] in the late stages of pregnancy between mid-July and early August 2007 from Daotanghe (36°26′N, 100°56′E), Qinghai, northwestern China. Females were transported to our laboratory in Hangzhou, where four or five were housed together in each 600 × 400 × 500-mm (length × width × height) cage with a substrate of moist sand (250 mm depth). All cages were placed in a room where temperatures varied between 22 and 28 °C. Thermoregulatory opportunities were provided during daylight hours by a 100-W incandescent lamp suspended over one end of the cage; overnight temperatures matched room temperatures. Mealworms (larvae of *Tenebrio molitor*), house crickets (*Acheta domesticus*) and water enriched with vitamins and minerals were provided ad libitum.

Females collected in early May were palpated every other day, and individuals that had just ovulated were assigned to one of six temperature treatments. We maintained 28 pregnant females in each of five 3 × 4 × 2-m artificial atmospheric phenomena simulator (AAPS) rooms to control their body temperatures at 24, 28, 29, 32 or 35 (±0.3) °C. We rotated cages within each AAPS room every other day according to a predetermined schedule to minimize any cage effects on results. The cloacal temperature was taken for each female using a UT325 digital thermometer (Shanghai Medical Instruments, China) to confirm that the body temperature was controlled at the intended level. The remaining 35 pregnant females [thermoregulating in the laboratory for 12 h daily (TR females)] were kept in a room with the temperature set at 18 °C. A 100-W heating light suspended above one end of the cage created a thermal gradient ranging from room temperature to 55 °C during the photophase under a 12:12-h light/dark cycle, allowing the TR females to thermoregulate for 12 h daily.

We checked the cages four times daily for newborns after the first female gave birth and collected and weighed them within 6 h of birth. Body mass and snout-vent length (SVL) were taken for each postpartum female. Females were isolated from each other using dividers that created 300 × 200 × 200-mm chambers if they gave birth during the same period in the same cage, so that newborns could be accurately allocated to the mother. None of these females was isolated for more than 36 h. Postpartum females were released at the site of capture in mid-September.

Thermal preference

An independent sample of 25 pregnant females, 25 non-pregnant females and 25 adult males (>65 mm SVL) collected in late July 2007 was used to examine whether females shift their thermal preferences when pregnant. The experiment was conducted under the conditions described above for the TR females. Five lizards, a random mix of pregnant females, non-pregnant females and adult males, were introduced into each cage at 0700 hours when the heating light was switched on. To remove the possible influence of diel variation in thermal preference, all measurements were started at 1500 hours and ended at 1700 hours. Body (cloacal) temperature was taken for each lizard using the same digital thermometer described above. Each lizard was measured three times, once on each of 3 consecutive days. The three measurements did not differ significantly (repeated measures ANOVA; $P > 0.518$ in all three groups), so we considered the mean of the three measurements to be a lizard's preferred body temperature (indicative of thermal preference).

Offspring phenotypes

On the day of birth newborns were first measured for sprint speed, and then for morphological traits. All running trials were conducted at a body temperature of 30 °C, which was controlled by placing the newborns in a Shellab incubator (Sheldon MFG, USA) at 30 °C for 30 min prior to testing. Sprint speed was assessed by chasing the neonates along a 2-m racetrack with one transparent side, which allowed videotaping with a NV-DS77 digital video camera (Panasonic, Japan). The racetrack was placed in a room maintained at 30 °C. Each newborn was chased twice, with a 30-min rest in the incubator between the two successive trials. The tapes were later examined for sprint speed in the fastest 250-mm interval with a computer using MGI VideoWave III (MGI Software, Canada).

Following the running trials, the newborns were moved into an AAPS room set at 4 °C and then measured with digital calipers (Mitutoyo, Japan). Measurements taken for each newborn included SVL, tail length, abdomen length (from the posterior base of the forelimb to the anterior base of the hind limb), head length (from the snout to the posterior end of the skull), head width (taken at the posterior end of the mandible), forelimb length (humerus plus ulna) and hind limb length (femur plus tibia). For forelimb and hind limb lengths, both sides were measured and a mean value was used. We determined neonate sex by gently pressing on both sides of tail base using forceps to check for the presence or absence of hemipenes.

Following morphological measurements, we randomly moved newborns into twelve 500 × 400 × 400-mm cages placed in a room set at 18 °C. Neonates were individually numbered at 15-day intervals with a non-toxic waterproof marker. A 60-W heating light mounted in each cage allowed thermoregulation for 12 h daily. Small mealworms and house crickets were provided in excess and spread throughout the cage, such that the newborns had free access to food. We evaluated early growth by weighing the offspring at 60 days of age.

Statistical analyses

We excluded the females at 24 and 28 °C from the analyses, because 100 % of the females at 24 °C and 93 % of the females at 28 °C failed to give birth or produced stillborns. A preliminary analysis revealed that none of the offspring phenotypes examined differed between the sexes, so data from the same litter were pooled to avoid pseudo-replication. We used repeated measures ANOVA with time as the within-subject factor and burrow as the between-subject factor to examine whether there was diel and spatial variation in air and burrow temperatures. We used linear regression analysis to examine whether an examined reproductive variable was related to maternal SVL, and whether an examined offspring trait was related to neonate SVL or mass. We used one-way ANOVA to examine whether pregnant females, non-pregnant females and adult males differed in thermal preference. We used one-way ANOVA and analysis of covariance (ANCOVA) (with maternal SVL as the covariate) to examine whether female reproductive traits differed among temperature treatments. We used one-way ANOVA and ANCOVA (with neonate size, SVL or mass as the covariate) to examine whether offspring phenotypes differed among temperature treatments. A principal component analysis (PCA) based on seven morphological variables (body mass, abdomen length, head length, head width, tail length, forelimb length and hind limb length) was used to show positions of neonates from different temperature treatments on a two-dimension plane; size effects were removed in all cases by using residuals from the regressions on neonate SVL. Tukey's host hoc test was performed on the traits that differed among treatments. All statistical analyses were performed with Statistica 6.0 (Tulsa, OK). Throughout this paper, values are presented as mean ± SE, and the significance level is set at $P = 0.05$.

Results

Surface temperatures overall varied from −4 to 64 °C (mean ≈16 °C) in May, 2–57 °C (mean ≈17 °C) in July, and 1–44 °C (mean ≈11 °C) in September. Burrow temperatures overall varied from 7 to 18 °C (mean ≈9 °C) in May, 13–17 °C (mean ≈14 °C) in July, and 10–15 °C (mean ≈11 °C) in September. On a daily basis, surface temperatures varied among the 24 time points ($P < 0.0001$ in all 3 months) but not among the four burrows ($P > 0.593$ in all 3 months), and burrow temperature varied among the four burrows ($P < 0.0001$ in all 3 months) but not among the 24 time points ($P > 0.825$ in all 3 months) (Fig. 1). Thermal fluctuations decreased at an ever decreasing rate as burrow depth increased and, at a depth of 600 mm, thermal fluctuations were almost negligible (Fig. 2). Pregnant females, non-pregnant females and adult males did not differ from each other in thermal preference ($F_{2,72} = 0.59$, $P = 0.557$) (Table 1).

Thirty-eight (95 %) NAT females, 31 (89 %) TR females, 18 (64 %) females at 29 °C, twenty-five (89 %) females at 32 °C, and 20 (71 %) females at 35 °C successfully gave birth, with none of them producing stillborns or deformed offspring (Table 2). Of the 44 deformed offspring, four were produced by one NAT female, 11 by three TR females, and 29 by seven females at 35 °C. The NAT females gave birth later than the TR females by an average of 20 days, and the TR females gave birth later than the females at 29 °C by an average of 5 days (Table 2). Postpartum body mass and litter size did not differ among the five temperature treatments after accounting for maternal SVL (Table 2). The SVL-specific mean neonate mass was greatest in the NAT treatment and lowest in the 29 °C treatment, as was the SVL-specific mean litter mass (Table 2).

All morphological traits except hind limb length examined in neonates differed among the five treatments (Table 3). The PCA resolved two components (eigenvalues ≥1) from seven SVL-adjusted morphological variables, accounting for 53.1 % of the variation in the original data (Table 4). The first component (31.5 % variance explained) had a high positive loading for forelimb length, and the second component (21.6 % variance explained) had a high negative loading for head width (Table 4). Neonates born in the five treatments differed in their scores on the first and

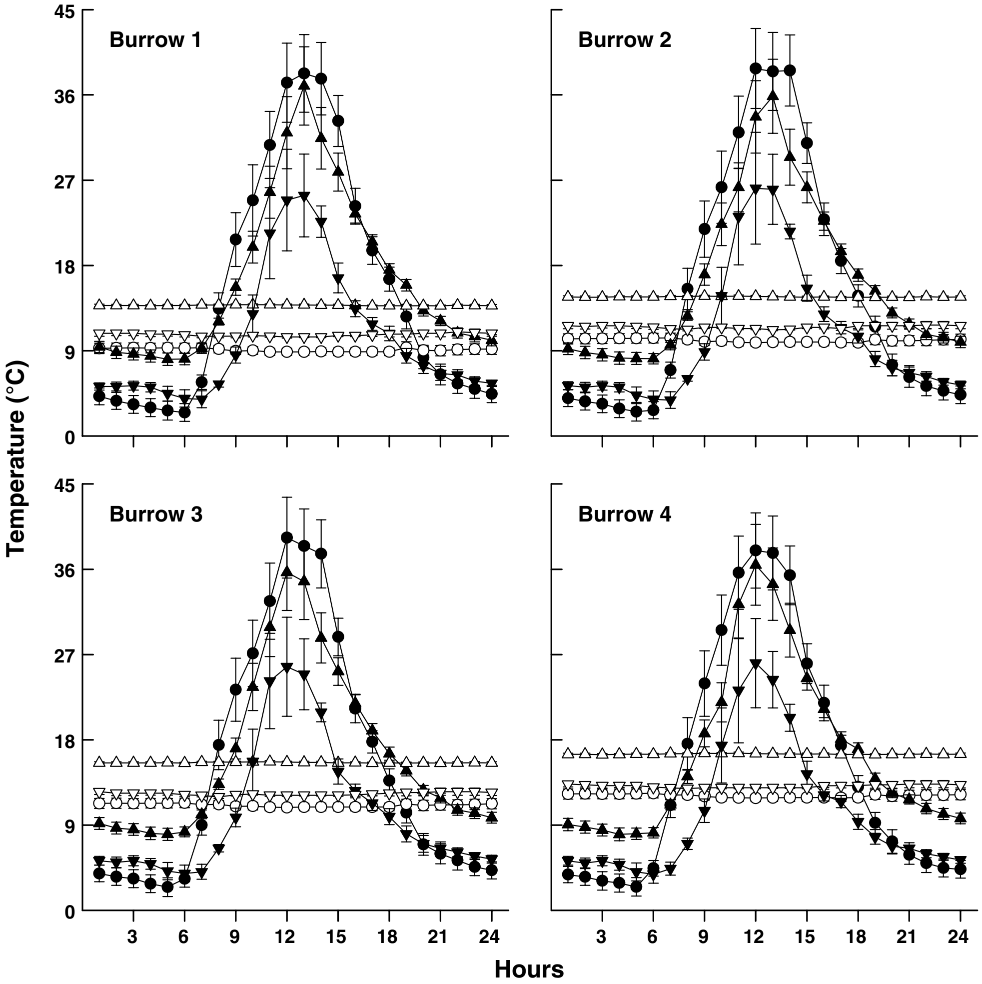

Fig. 1 Mean surface (10 mm above the ground) and burrow (600 mm below the ground) temperatures recorded in the field. *Solid dots* Surface temperatures in May, *solid triangles* surface temperatures in July, *solid inverted triangles* surface temperatures in September, *open dots* burrow temperatures in May, *open triangles* burrow temperatures in July, *open inverted triangles* burrow temperatures in September

second axes (Table 4). Overall, neonates produced by the NAT and TR females differed morphologically from those produced by the females at 29 and 32 °C, while neonates produced by the females at 35 °C did not differ significantly from those born in the other four treatments (Fig. 3; Table 4).

Neonates born in the five treatments differed in sprint speed ($F_{4,\,127} = 5.71$, $P < 0.0003$), with the fastest neonates produced by the NAT females and slowest neonates produced by the females at 29 °C (Fig. 4). All neonates measured on the day of birth survived to 60 days of age. Mass gain in the first 60-day period was positively related to mass at birth ($P < 0.005$ in all five treatments). Offspring born in the five treatments differed in body mass at 60 days of age ($F_{4,\,126} = 6.38$, $P < 0.0002$), with neonates produced by the females at 29 °C growing more rapidly than those born in the other four treatments, accounting for body mass at birth (Fig. 5).

Discussion

In agreement with studies of other viviparous lizards (Ji et al. 2006, 2007; Li et al. 2009; Tang et al. 2012) and snakes (Webb et al. 2006; Gao et al. 2010), our data showed that the thermal environment experienced by pregnant females affected a number of offspring traits (size, mass, morphology, locomotor performance and early growth) tightly associated with fitness in many species of lizards. Morphologically, neonates could be divided into three groups, those born in the NAT and TR treatments, in the 29 and 32 °C treatments, and in the 35 °C treatment (Table 4; Fig. 3). However, of the morphometric traits used to divide neonates, only two (forelimb length and head width) contributed most to positions of neonates on a two-dimension plane (Table 4). Neonate mass and sprint speed both were greatest in the NAT treatment and smallest in the 29 °C treatment, with

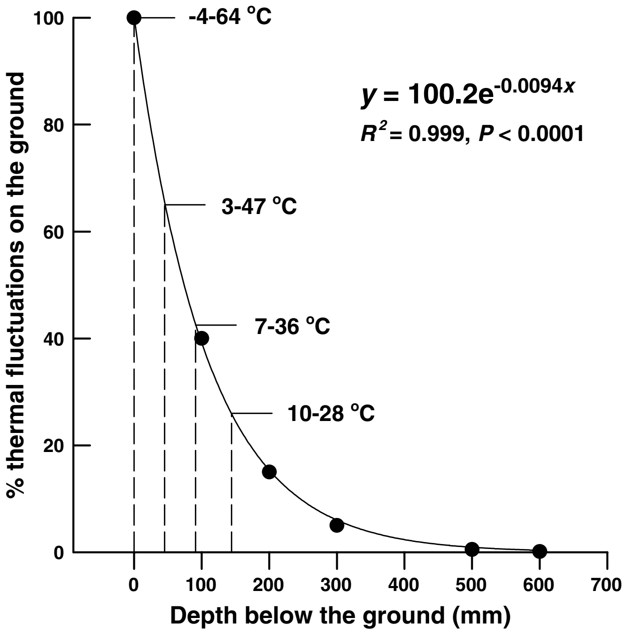

Fig. 2 A curvilinear relationship between percentage of thermal fluctuations on the ground surface and burrow depth, according to data from Zhang (2006) (at depths of 100, 200, 300 and 500 mm) and this study (on the ground surface and at a depth of 600 mm)

Table 1 Body temperatures (°C) preferred by pregnant females, non-pregnant females and adult males on the laboratory thermal gradient

	n	Mean	SE	Range
Pregnant females	25	34.4	0.2	32.0–36.1
Non-pregnant females	25	34.7	0.3	31.3–37.0
Adult males	25	34.3	0.3	31.7–37.1

the other three treatments in between (Table 2; Fig. 4). These results suggest relatively high reproductive fitness in the NAT females, as larger and faster offspring often have better performance and higher survival (Ferguson and Fox 1984; Congdon et al. 1999; Janzen et al. 2000a, b; Warner and Andrews 2002; Husak 2006). Contrary to what was expected, neonates produced at 29 °C grew more rapidly than those born in the other four treatments (Fig. 5). This result showed that slower neonates diverted more acquired energy to growth. Alternatively, it was possible that neonates produced at 29 °C compensated for their smaller sizes at birth by 'catch-up growth'. The rate of postnatal or post-hatching growth does not always depend on size at birth or hatching (Dibattista et al. 2007; Ji et al. 2009a), and this is especially true for young individuals kept in the laboratory that have free access to food (Ji et al. 2003, 2006; Li et al. 2009, 2011, 2013; Gao et al. 2010). Besides the above findings, this study showed five major results.

First, long-term exposure of pregnant *P. vlangalii* to temperatures outside the range of 29–35 °C resulted in the failure of embryonic development. In a wide range of reptile taxa the temperature response of embryonic survivorship exhibits an inverted, left skewed U-shape, with sharp thresholds at low and high temperatures and relatively invariant survivorship at intermediate temperatures (Ji et al. 2003, 2006; Du et al. 2010; Gao et al. 2010; Lin et al. 2010; Li et al. 2012). This pattern suggests that temperature has a nominal effect on embryonic survivorship except in determining the lower and upper limits for viability. The range of temperatures that do not differentially affect embryonic survivorship varies among and within species, being generally correlated with a species' or population's natural thermal environment (Lin et al. 2010 and references therein). Low temperatures slow or even arrest embryonic development but often have little or no lethal effect on embryos, while extremely high temperatures have a lethal effect on embryos or increase embryonic morbidity (Deeming 2004). At the time intervals that did not allow behavioral thermoregulation, the TR females were exposed to the temperature of 18 °C, and the NAT females to temperatures lower than 18 °C (Fig. 1). Interestingly, 89 % of the TR females and 95 % of the NAT females successfully gave birth. Of the 44 deformed offspring, 29 were produced by the females at 35 °C, and the remaining 15 by the NAT and TR females that had the possibility to encounter temperatures higher than 35 °C. Taken together, the above findings provide three inferences regarding embryonic thermosensitivity in *P. vlangalii*: long-term exposure of pregnant females to temperatures outside the range of 29–35 °C may result in the failure of embryonic development, daily or short-term exposure of pregnant females to temperatures lower than 29 °C may not necessarily increase embryonic mortality, and daily or short-term exposure of pregnant females to temperatures higher than 35 °C may increase embryonic morbidity.

Second, the rate of embryonic development was similar between *P. vlangalii* and congeneric oviparous species. The influence of incubation temperature on embryonic development has been examined only in two oviparous *Phrynocephalus* lizards, *P. frontalis* and *P. versicolor*. Eggs of these two species do not differ in incubation length when kept at the same temperature, with the mean incubation period being 45 days at 24 °C, 32 days at 28 °C, and 24 days at 32 °C (Qu et al. 2011). Moreover, embryos of the two species identified at oviposition are at Dufaure and Hubert's (1961) stages of 30–32, with a mean stage of 31 (Qu et al. 2011), which suggests that they are among the oviparous lizards where the length of intrauterine development accounts for about 1/3 of the whole period of embryonic development (Shine 1983). Accordingly, we may expect that in the two oviparous species the whole period of

Table 2 Reproductive traits of *Phrynocephalus vlangalii* females under five temperature regimes

	Temperature treatments					Results of statistical analyses
	NAT	TR	29	32	35	
n	38	31	18	25	20	
SVL (mm)	72.2 ± 0.6 64.8–79.5	69.0 ± 0.8 61.2–78.3	67.0 ± 1.2 60.4–74.9	69.5 ± 0.8 60.7–77.0	68.8 ± 1.0 61.1–80.1	$F_{4,127} = 5.31$, $P < 0.001$ NAT^{a}, TR^{b}, 29^{b}, 32^{ab}, 35^{b}
Gestation length (days)	69.5 ± 1.0 60–85	49.9 ± 0.8 41–61	44.9 ± 0.6 42–51	37.8 ± 0.4 34–41	30.9 ± 0.4 28–34	$F_{4,127} = 259.75$, $P < 0.0001$ NAT^{a}, TR^{b}, 29^{c}, 32^{d}, 35^{e}
Postpartum mass (g)	11.0 ± 0.3 8.3–14.7	9.8 ± 0.3 6.7–12.4	9.1 ± 0.4 7.0–13.3	9.8 ± 0.3 6.5–12.4	9.7 ± 0.3 7.2–13.1	$F_{4,126} = 0.61$, $P = 0.656$
Litter size	4.4 ± 0.2 2–6	4.0 ± 0.2 2–6	3.0 ± 0.2 2–5	4.0 ± 0.2 2–6	3.4 ± 0.4 2–7	$F_{4,126} = 2.34$, $P = 0.059$
Neonate mass (g)	1.18 ± 0.02 0.86–1.57	1.05 ± 0.02 0.77–1.23	0.91 ± 0.04 0.55–1.20	1.05 ± 0.03 0.67–1.29	1.02 ± 0.05 0.63–1.39	$F_{4,126} = 8.20$, $P < 0.0001$ NAT^{a}, TR^{b}, 29^{c}, 32^{b}, 35^{bc}
Litter mass (g)	5.21 ± 0.22 2.28–7.85	4.22 ± 0.26 2.10–7.29	2.78 ± 0.28 1.10–5.16	4.18 ± 0.25 1.92–6.40	3.35 ± 0.32 1.94–6.34	$F_{4,126} = 7.01$, $P < 0.0001$ NAT^{a}, TR^{ab}, 29^{c}, 32^{ab}, 35^{b}

Only females that gave birth without any stillborns or deformed offspring were used. Values are expressed as mean ± SE and range. *F*-values of one-way ANOVA [for snout–vent length (*SVL*) and parturition date] or analysis of covariance (ANCOVA) (for postpartum mass, litter size, neonate mass and litter mass with SVL as the covariate, and for relative litter mass with postpartum mass as the covariate) are given in the table

NAT Females thermoregulating under natural conditions, *TR* females thermoregulating in the laboratory for 12 h daily, *29* females kept at 29 °C, *32* females kept at 32 °C, *35* females kept at 35 °C

[a, b, c, d, e] Treatments with *different superscripts* differ significantly (Tukey's post hoc test, α = 0.05; a > b > c > d > e)

Table 3 Size and morphology of neonates produced by female *P. vlangalii* under five temperature regimes

	Temperature treatments					Results of statistical analyses
	NAT	TR	29	32	35	
n	38	31	18	25	20	
SVL (mm)	30.9 ± 0.4 25.5–34.9	27.4 ± 0.3 24.0–30.3	28.9 ± 0.4 25.6–31.7	29.1 ± 0.6 23.9–34.1	30.8 ± 0.3 28.3–33.6	$F_{4,127} = 14.32$, $P < 0.0001$ NAT^{a}, TR^{d}, 29 ^{cd}, 32^{bc}, 35^{ab}
Body mass (g)	1.23 ± 0.02 1.03–1.60	1.04 ± 0.03 0.68–1.34	1.00 ± 0.03 0.79–1.22	1.05 ± 0.04 0.61–1.444	1.07 ± 0.03 0.84–1.35	$F_{4,126} = 16.04$, $P < 0.0001$ NAT^{a}, TR^{a}, 29^{bc}, 32^{b}, 35^{c}
Abdomen length (mm)	15.4 ± 0.3 10.3–18.3	13.2 ± 0.2 10.7–15.5	14.6 ± 0.3 11.9–16.2	14.4 ± 0.4 11.3–18.0	15.9 ± 0.1 13.9–16.9	$F_{4,126} = 4.11$, $P < 0.004$ NAT^{b}, TR^{a}, 29^{a}, 32^{ab}, 35^{a}
Head length (mm)	9.5 ± 0.2 6.8–10.9	8.3 ± 0.1 7.6–9.1	8.7 ± 0.2 7.5–9.4	9.1 ± 0.2 7.5–10.4	9.5 ± 0.1 8.7–10.1	$F_{4,126} = 2.84$, $P = 0.027$ NAT^{ab}, TR^{ab}, 29^{b}, 32^{a}, 35^{ab}
Head width	7.5 ± 0.1 6.2–8.5	7.1 ± 0.1 6.3–7.8	7.1 ± 0.1 6.5–7.7	7.0 ± 0.1 6.1–7.6	7.3 ± 0.1 6.5–8.1	$F_{4,126} = 5.90$, $P < 0.0003$ NAT^{ab}, TR^{a}, 29^{bc}, 32^{c}, 35^{bc}
Tail length (mm)	27.7 ± 0.2 23.0–30.2	25.3 ± 0.4 19.8–30.0	25.2 ± 0.4 22.0–28.0	26.4 ± 0.6 19.0–30.8	28.1 ± 0.4 25.0–33.0	$F_{4,126} = 5.06$, $P < 0.001$ NAT^{a}, TR^{a}, 29^{b}, 32^{a}, 35^{a}
Forelimb length (mm)	10.0 ± 0.1 8.4–10.8	9.1 ± 0.1 7.5–10.3	9.1 ± 0.1 7.6–10.4	9.0 ± 0.1 7.3–10.3	9.8 ± 0.1 8.9–10.6	$F_{4,126} = 8.66$, $P < 0.0001$ NAT^{a}, TR^{ab}, 29^{bc}, 32^{c}, 35^{ab}
Hind limb length (mm)	14.1 ± 0.1 11.6–15.5	12.9 ± 0.2 10.7–14.4	13.2 ± 0.1 12.4–14.2	13.2 ± 0.3 9.9–15.2	14.2 ± 0.1 13.1–15.2	$F_{4,126} = 2.26$, $P = 0.066$

Only neonates produced by females that gave birth without any stillborns or deformed offspring were used. Values are expressed as mean ± SE and range. *F*-values of one-way ANOVA (for SVL) or ANCOVA (for the remaining variable with SVL as the covariate) are given in the table. For abbreviations, see Table 2

[a, b, c] Treatments with *different superscripts* differ significantly (Tukey's post hoc test, α = 0.05; a > b > c)

embryonic development is about 68 days at 24 °C, 48 days at 28 °C, and 36 days at 32 °C. This relationship between the developmental rate (y) and temperature (x) can be well described by the equation $y = 474.1e^{-0.08x}$ ($R^2 = 0.997$, $P < 0.01$), with which we can predict that the whole period of embryonic development is about 47 days at 29 °C, 37 days at 32 °C, and 29 days at 35 °C. These predicted values are respectively close to mean gestation lengths

Table 4 Loading of the first two axes of a principal component (*PC*) analysis on seven offspring morphological variables measured at birth

	Factor loading	
	PC1	PC2
Body mass	0.528	−0.360
Abdomen length	−0.560	−0.437
Head length	−0.337	−0.699
Head width	−0.050	*−0.782*
Tail length	0.666	−0.097
Forelimb length	*0.785*	−0.121
Hind limb length	0.660	−0.256
Variance explained (%)	31.5	21.6

Factor scores on PC1: $F_{4,\,127} = 7.13$, $P < 0.0001$; NAT^{a}, TR^{a}, 29^{b}, 32^{b}, 35^{ab}

Factor scores on PC2: $F_{4,\,127} = 3.28$, $P = 0.014$; NAT^{ab}, TR^{b}, 29^{a}, 32^{ab}, 35^{ab}

Size effects are removed in all cases by using residuals from the regressions on neonate SVL. Variables with the main contribution to each factor are *in italics*. For abbreviations, see Table 2

[a, b] Treatments with *different superscripts* differ significantly (Tukey's post hoc test, α = 0.05; a > b)

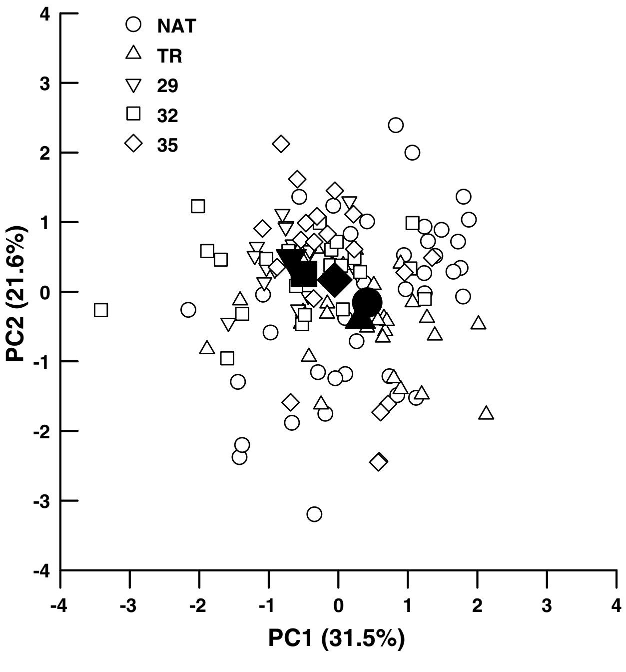

Fig. 3 Positions of *Phrynocephalus vlangalii* neonates produced by females under five temperature regimes in the space defined by the first two axes of a principal component (*PC*) analysis based on seven morphological variables. Size effects were removed using residuals from the regressions of corresponding variables on snout-vent length. *Larger black symbols* show the mean values of scores on the two axes. *NAT* Females thermoregulating under natural conditions, *TR* females thermoregulating in the laboratory for 12 h daily, *29* females kept at 29 °C, *32* females kept at 32 °C, *35* females kept at 35 °C

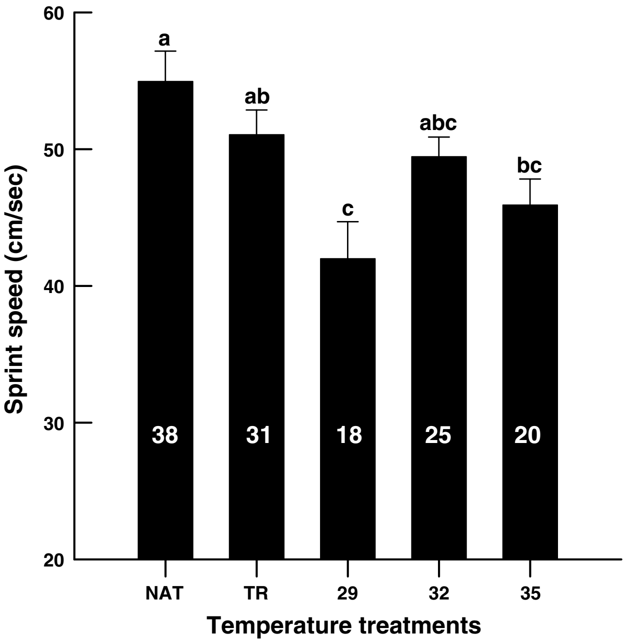

Fig. 4 Sprint speed of neonates produced by *P. vlangalii* females under five temperature regimes. Means with *different letters* differ significantly (Tukey's post hoc test, α = 0.05; a > b > c). *Numbers inside bars* indicate sample size for each temperature treatment. See Fig. 3 for abbreviations

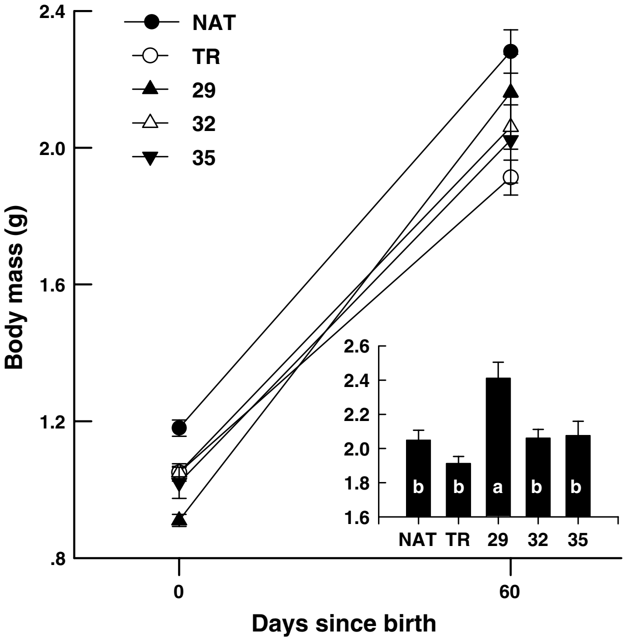

Fig. 5 Body mass at birth and 60 days of age. The *inset* shows adjusted mean values (+SE) for body mass at 60 days of age, with mass at birth set at 1.05 g. Adjusted means with *different letters* differ significantly (Tukey's post hoc test, α = 0.05; a > b). See Fig. 4 for sample size for each treatment. See Fig. 3 for abbreviations

at 29 °C (45 days), 32 °C (38 days) and 35 °C (31 days) recorded in this study (Table 2), allowing the conclusion that the rate of embryonic development is similar between *P. vlangalii* and congeneric oviparous species.

Third, *P. vlangalii* females did not shift their thermal preferences when pregnant. Pregnant females should avoid low body temperatures, because lengthy gestation lengths at low body temperatures increase reproductive costs associated with decreased survival or future reproduction of females by increasing the time over which these costs are incurred. Pregnant females should also avoid high body temperatures, because the increased metabolic rates at high body temperatures increase energetic costs and hence reproductive costs. Thus, the maximization of reproductive benefits should be achieved in pregnant females by shifting thermal preferences towards the levels optimal for embryonic development and meanwhile minimizing reproductive costs (Lin et al. 2008). In viviparous lizards where the rate of embryonic development increases at an ever decreasing rate as gestation temperature increases, pregnant females often maintain lower than usual body temperatures because they benefit increasingly less from keeping higher body temperatures in terms of the reduced gestation length (Ji et al. 2006, 2007; Lin et al. 2008; Li et al. 2009; Tang et al. 2012). In this study, females did not maintain cooler body temperatures when pregnant, as revealed by the fact that pregnant females, non-pregnant females and adult males did not differ from each other in thermal preference (Table 1). Our explanation for this result is that keeping body temperatures at the mean level (34.4 °C) close to the upper thermal limit for embryonic development allows pregnant *P. vlangalii* to produce high-quality offspring in the shortest possible time. This strategy could be ecologically important for high-altitude lizards such as *P. vlangalii* that have more limited opportunities to thermoregulate.

Fourth, short-term gestation temperatures below the lower thermal limit for successful embryonic development slowed but did not arrest development in *P. vlangalii*. Though having the potential to maintain body temperatures at a mean level close to 35 °C for about 12 h daily (Table 1), the TR females gave birth later than did the females at 29 °C by an average of 5 days (Table 2). This difference indicated that the overall mean body temperature was lower than 29 °C in the TR females, and that temperatures as low as 18 °C slowed but did not arrest embryonic development. It has been documented in adult *P. vlangalii* that the cooling rate is slightly greater than 0.25 °C/min (Shu et al. 2010). Accordingly, the TR females could be expected to cool from 34.4 °C (the mean level of thermal preference) to 18 °C (the temperature to which they were exposed when the heating light was switched off) in less than 1 h. Thus, if embryonic development were arrested at 18 °C, the TR females would give birth much later than the date recorded because their embryos could develop at a mean temperature of 34.4 °C for at most 12 h daily. The NAT females gave birth later than the TR females by an average of 20 days (Table 2). This difference resulted from two factors: mean temperatures in natural burrows were lower than 18 °C (Fig. 1); and at the study site pregnant females could thermoregulate for no more than 9 h, because even on cloudless summer days daily activity is restricted to the time period of 0900–1800 hours (Zhang 2006).

Fifth, *P. vlangalii* embryos in nature had no means of completing development without reliance on maternal thermoregulation. The areas occupied by viviparous *Phrynocephalus* lizards are characterized by a low mean but great range of thermal fluctuations. As in other reptile burrows or nests (Bodie et al. 1996; Demuth 2001; Booth 2006), the range in daily thermal fluctuations decreases at an ever decreasing rate as depth increases in *P. vlangalii* burrows (Fig. 2). At a depth of 600 mm daily fluctuations of temperature were almost negligible and the highest temperature (17.5 °C) ever recorded at this depth was far below the lower thermal limit for embryonic development (Fig. 1). Based on the curvilinear relationship between the greatest range (from −4 to 64 °C) in daily thermal fluctuations on the ground surface and burrow depth (Fig. 2), we can expect that *P. vlangalii* embryos cannot develop at depths deeper than 150 mm because the highest temperatures at these depths are lower than 28 °C to which prolonged exposure of embryos may result in the failure of development. Is it possible for *P. vlangalii* females to find shallower burrows or to stay at shallower levels where thermal conditions are appropriate for embryonic development? Our answer to this question is no, because such a possibility, if present, is rare or ephemeral. In many shallow reptile burrows or nests (<300 mm depth) there is little difference (<0.5 °C) in daily mean temperature at different levels below the ground surface (Booth 2006). Temperatures higher than 47 °C are lethal to adult *P. vlangalii* (Shu et al. 2010). Accordingly, individuals staying at depths shallower than 45 mm had the possibility to encounter high temperatures potentially lethal to them (Fig. 2). Figure 2 shows that thermal conditions at depths ranging from about 90 to 150 mm potentially allow embryonic development to take place, because temperatures higher than 28 °C but lower than 36 °C are appropriate for embryonic development. However, such thermal conditions are unlikely to persist in nature because both the mean and the magnitude of thermal fluctuations are not invariant and predictable. Due to the nature of thermal fluctuations and low thermal means in natural burrows, it is impossible for *P. vlangalii* females to find any site-fixed burrows inside which embryos can complete development without relying on maternal thermoregulation.

In summary, our data demonstrate that long-term exposure of pregnant *P. vlangalii* to temperatures outside the range of 29–35 °C may result in the failure of embryonic development, and that short-term low gestation temperatures slow but do not arrest embryonic development. The developmental zero temperature at which embryonic development is effectively arrested is currently unknown, but it must be lower than 18 °C. Unlike females of some viviparous lizards, pregnant *P. vlangalii* do not shift their thermal preferences downwards, but try to maintain body temperatures at a mean level very close to the upper thermal limit for successful embryonic development when conditions allow them to do so. This strategic thermal preference allows pregnant *P. vlangalii* to produce offspring in the shortest possible time. Mean temperatures inside natural burrows are far below the lower thermal limit for successful embryonic development, and females in nature cannot find any site-fixed burrows inside which thermal conditions are appropriate for embryonic development. Our data validate the hypothesis that viviparity in high-altitude *Phrynocephalus* lizards is adaptive because embryos cannot complete development without relying on maternal thermoregulation.

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