Why do female desert lizards construct burrows to lay eggs?

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Abstract

Many oviparous animals construct well-designed nests to provide relatively favourable conditions for their eggs and hatchlings, but the direct evidence that nest structure can determine their reproductive success is insufficient. In the present study, we explored the structure of nests and its effect on nest environments and reproductive success in the toad-headed agama (*Phrynocephalus przewalskii*). We observed that female *P. przewalskii* constructed burrow nest consisting of an inclined tunnel and an expanded chamber. We constructed artificial nests with or without the burrow to determine how burrows influence nest environments, egg survival and successful emergence of hatchlings. Our results indicated that burrow nests had higher and more stable humidity than non-burrow nests. More importantly, egg survival and the emergence success of hatchlings were significantly higher for burrow nests than for non-burrow nests. Therefore, our manipulation experiments provide direct evidence that maternal nest construction behaviour could determine parental reproductive success in reptile.

Keywords

egg survival, embryonic development, emergence success, maternal effect, nest structure, oviposition behaviour.

1. Introduction

The developmental trajectory of embryos may be shaped by environmental conditions (e.g., temperature and oxygen), and can have a profound impact

on the fitness of individuals in post-embryonic life history stages (Deeming, 2004; Gorman & Nager, 2004). Maternal effect and its interaction with the environment are considered one of the most important contributions to environmental conditions experienced by embryos (Bernardo, 1996; Mousseau & Fox, 1998). Unlike viviparous animals, which can provide stable development environments for their embryos, the embryos of oviparous animals are directly exposed, and vulnerable, to the unstable external environment. Adaptive maternal oviposition behaviour (e.g., nest site selection, nest construction) is thus expected to protect embryos from the effects of unpredictable environmental conditions (Refsnider & Janzen, 2010; Du & Shine, 2015), given that it is the primary determinant of embryonic developmental conditions in oviparous animals (Bernardo, 1996).

Many oviparous animals construct well-designed nests to provide relatively favourable conditions for their eggs and developing hatchlings (Hansell, 2007), from simple underground reptile nests, to elaborate domed bird nests, and sophisticated ant nests (Thompson et al., 1996; McGowan et al., 2004; Tschinkel, 2004). Ecological adaptation in nest construction behaviour is an important aspect of animal nesting research and has attracted a great deal of scientific attention (Chapuisat et al., 2007; Japoshvili et al., 2012; Møller & Nielsen, 2015; Refsnider, 2016). The primary function of animal nests is to modify the nest environment (e.g., temperature, humidity, and oxygen) in a way that can enhance the survival of eggs and offspring. For example, nests can minimize heat loss of eggs and hatchings (Heenan, 2013) and keep them protected from extreme temperatures (Seidelmann, 1999). In addition, many animals build nests in order to reduce risk of predation and parasitism and thereby increase reproductive success (Chapuisat et al., 2007; Kreisinger & Albrecht, 2008). However, there are still knowledge gaps in this field. One of the reasons for this is that research on ecological adaptation of nest construction behaviour is plagued by taxonomic bias. Avian studies have dominated the scientific literature, although studies on other lineages are increasing (Barber, 2013). Furthermore, while the important role of nest site and structure in determining physical environments is known in a number of species, the ecological consequence (e.g., reproductive success) of nest construction has not been explicitly demonstrated in many cases (Wiebe, 2001; Mennerat et al., 2009).

Many reptiles, such as tuatara, turtles and some lizards (Thompson et al., 1996; Matsuzawa et al., 2002; Iverson et al., 2004; Ryberg et al., 2012), construct burrows or cavities to lay their eggs. Unlike birds, most oviparous

reptiles construct nests and leave their eggs alone without parental care, while their mating and oviposition are discrete events, separated temporally and spatially (Angilletta et al., 2009; Refsnider, 2016). The effect of nest structure on reproductive success is thus not coupled with mate choice and parental care effects. Furthermore, the reproductive success of oviparous reptiles is highly dependent on nest physical conditions (e.g., temperature, water, and oxygen), because these environmental factors can significantly influence embryonic development and survival, offspring phenotypes, and fitness in reptiles (Deeming, 2004; Booth, 2006). Therefore, oviparous reptiles are excellent models for detecting how nest structure influences reproductive success.

In this study, we explored the structure of nests and its effect on nest environments (temperature, moisture and oxygen concentration) and reproductive success in the toad-headed agama (*Phrynocephalus przewalskii*). We found that *P. przewalskii* dug burrows for their eggs, and we investigated the nest structure in the field. Then, we constructed artificial burrow and non-burrow nests, to incubate *P. przewalskii* eggs and determine nest environments, and thereby tested two hypotheses for the function of burrow nests: (1) the burrows enhance egg survival because nest structure may optimize incubation conditions; (2) the burrows increase the emergence success of hatchlings, an important component of reptilian reproductive success (Tomillo et al., 2009), because *P. przewalskii* eggs are laid in relatively deep underground nests.

2. Methods

2.1. Study location and species

The toad-headed agama (*Phrynocephalus przewalskii*) is a small agamid lizard (adult snout–vent length 44–59 mm), generally inhabiting desert, semi-desert or grassland habitats (Zhao et al., 1999). Females typically produce one or two clutches of eggs, from late May to late July. In the field, gravid females dig burrows in the sand to lay their eggs (Figure 1a).

In our study site at Ordos Field Observation Station on Sandy Grassland (Institute of Grassland Research of the Chinese Academy of Agricultural Sciences; Ministry of Agriculture, P.R. China, 40°12′N, 111°07′E; elevation 1036 m), *P. przewalskii* is very common in habitats mainly covered by the
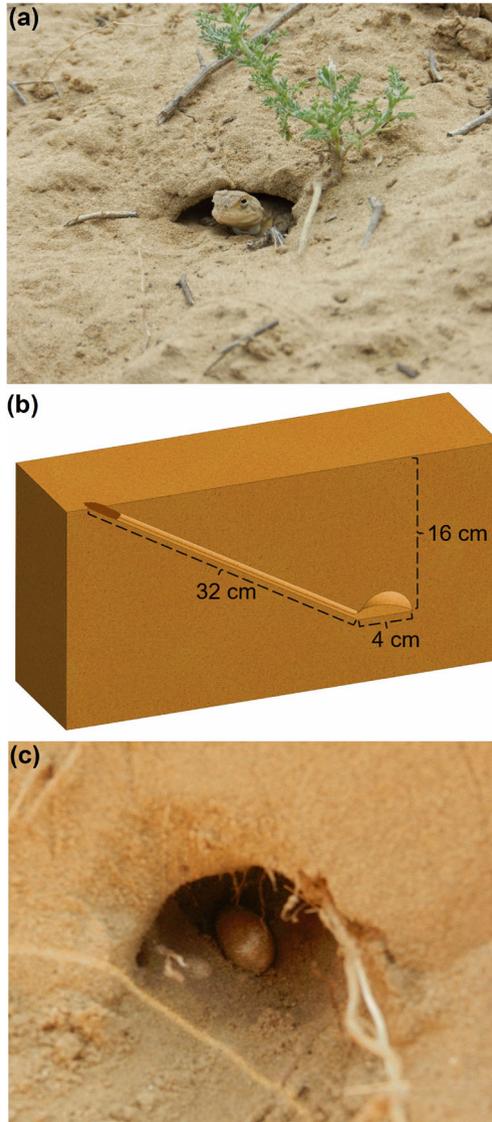
Figure 1. Burrows of *Phrynocephalus przewalskii*. (a) A female *P. przewalskii* at the entrance to its burrow; (b) diagram of the structure of one typical burrow (side view). The burrow entrance was plugged with sand, but the tunnels and chambers were not; (c) eggs in the nest chamber.

shrub *Artemisia ordosica*. The study site is located in an arid zone, with average annual precipitation of 300 to 380 mm, and average annual temperature of 7.8°C.

2.2. Experiment procedures

We located *P. przewalskii* nests in the field and excavated them to determine the nest structure. To determine the influence of nest structure on nest environments, we constructed two artificial nests (with and without nest structure) near each maternally selected nests and monitored the nest environments. At the same time, we collected eggs of gravid *P. przewalskii* in our laboratory and incubated eggs in another two artificial nests (with and without nest structure) to determine the influence of nest structure on embryonic development and hatchling traits.

2.3. Nest structure

During the reproductive season in 2015, we tracked gravid *P. przewalskii* in the field to locate their nests. The nest of *P. przewalskii* was an inclined burrow in the sand, with a small chamber at the end of the burrow where the eggs were laid. Once a nest was located, we excavated the burrow using a plastic spoon. To determine the structure of the nest, we measured the width, height, depth, and length of the burrows at two cross sections located at the front and middle parts of the burrow. The width, height, and length of the chamber at the end of the burrow were also measured. Totally, 34 nests were excavated to determine the nest structure.

2.4. Influence of nest structure on nest environments

We located another 13 maternally selected nests in our field sites to determine the influence of nest structure on nest environments. Near each maternally selected nest (within 20 cm), we constructed two artificial nests (10 cm apart), at the same depth as the natural nest. In each artificial nest, we carved out a chamber using a trowel and spoon. One of the artificial nests was then constructed as a burrow nest by placing a plastic corrugated tube (2.5 cm diameter, 25 cm length) in the sand to simulate the burrow of a maternally selected nest. The entrance of the tube was plugged with a piece of cotton mixed with sand. The other artificial nest was backfilled with sand to create a non-burrow nest without the burrow structure. We then monitored temperature, humidity, soil moisture and oxygen concentration in these real

and artificial nests on an average of 33 days, covering roughly 82.5% of the incubation duration.

Of the 13 real nests, six nests were excavated to determine nest temperature, humidity and soil moisture. An iButton data logger (DS1923-F5, Maxim Integrated Products, San Jose, CA, USA) was placed in the chamber at the end of each nest to record temperature and relative humidity hourly. Every three days, we collected about 10 g soil from the nest chambers to determine soil moisture (SM). We weighted the wet mass (WM) of soil samples immediately and then determined its dry mass (DM) by oven-drying the soil at 105°C for 24 h. The soil moisture was calculated by the following formula: $SM = (WM - DM)/WM \times 100\%$. When restoring a nest after each excavation, we buried a smooth plastic tube (2.5 cm diameter, 35 cm length) that was then removed with caution to keep the burrow structure. The entrance of the nests was plugged with a piece of cotton mixed with sand. A same procedure was applied to determine the temperature, relative humidity and soil moisture in the artificial nests (burrow and non-burrow nests).

The other seven real nests and the associated artificial nests were used to determine oxygen concentration in nests. Two eggs were buried in each real nest and the two associated artificial nests. A slender plastic tube (0.25 cm diameter, 25 cm length) was buried beside each nest to sample gas available to eggs. The one end of the tube connected to the eggs in the nests, and the other end extended to the ground surface and was closed with a clip (Booth, 1998). We sampled 20-ml gas from each tube with a syringe every three days and measured oxygen concentration immediately by a portable digital oxygen analyser (CY-12C, Meicheng Electrochemical Analytical Instruments Factory, Hangzhou, P.R. China).

2.5. Influence of nest structure on embryonic development and hatchling traits

2.5.1. Female husbandry and egg collection
From late May to mid-July 2015, 18 gravid females were collected by hand. These females were housed individually in terraria ($31 \times 21 \times 18$ cm) at the laboratory of our field station. The bottoms of the terraria were filled with a 3-cm layer of moist sand, where females laid their eggs. Food (mealworms and crickets), and water were provided ad libitum. Lizards were exposed to natural photoperiod, and provided with additional heat via 45-W heat mats under the terraria, from 08:00 to 17:00 h. We checked terraria four times

daily for freshly laid eggs, which were weighed (±0.001 g) immediately. Following oviposition, lizards were released to the sites where they were caught.

2.5.2. *Nest manipulation experiments*

For these experiments, two eggs from each clutch were used. Eggs were placed individually in plastic cups with moist sand and transferred to the nesting area. We constructed another two artificial nests (burrow and non-burrow nests) beside each real nest ($N = 18$) as described above. Two eggs from the same clutch were separately buried in the chamber of the two artificial nests.

After the eggs had developed in the burrow and non-burrow nests for about 34 days, we excavated the nests, and recorded egg survival. Then the nests were restored and protected with 0.2-cm-mesh wire hardware cloth. Subsequently, we recorded hatchling emergence four times daily, and excavated nests to check for egg survival every other day. This procedure allowed us to determine hatching and emergence success and estimate the exact date of hatching in the artificial nests. Hatching success was defined as the percentage of eggs successfully hatched in the nests. Successful emergence was recorded once hatchlings emerged on the ground or reached the entrance of the burrow. As the hatchlings from the burrow nests reached to the ground within one or two days, we recorded hatchlings failing to reach the ground within three days as unsuccessful emergence in non-burrow nests. The hatchlings from both burrow and non-burrow nests were then taken to the laboratory and the snout–vent length (SVL), tail length (TL) and body mass (BM) were measured. Hatchlings were then released to where their mother were caught.

Research was performed under approvals from the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

2.6. *Statistical analyses*

The angle of the tunnel inclination was calculated by the formula $\theta =$ arcsin(depth/distance from the entrance to the cross section). We used Wilcoxon matched pairs test to detect the change in the dimension of burrows at different cross sections in real nests. From the data collected by the iButtons, we calculated the mean daily mean, mean daily maximum, mean daily minimum, and mean daily range of temperature and relative humidity. Then we

used one-way mixed-model ANOVA to compare these environmental variables with nest type (real nest, artificial burrow and non-burrow nests) as a fixed factor and block (each block includes one real nest and two associated artificial nests) as a random factor. Two-way mixed-model ANOVAs were used to detect the differences of soil moisture and oxygen concentration among nests, with date and nest type as fixed factors and block as a random factor. Fisher's exact tests were used to evaluate the effects of nest structure on hatching and emergence success. We used one-way ANOVA or ANCOVA, with egg mass as covariance, to test differences between treatments in incubation duration and hatchling morphological traits. Prior to parametric analyses, data were tested for normality using the Shapiro–Wilk test, and for homogeneity of variances using Levene's test. Soil moisture was arcsine square root transformed prior to statistical analyses. Data were expressed as mean $\pm$ SE.

3. Results

3.1. Nest structure

Phrynocephalus przewalskii females constructed burrows with a mean depth of 14.6 ± 0.4 cm ($N = 34$), consisting of an inclined tunnel and an expanded chamber at the end (Figure 1b). Eggs were half-buried by females in the sand within the air–filled chamber (Figure 1c). The entrances of burrows were loosely covered by sand (<5 cm), but the tunnels and chambers were not. The inclination angle for the tunnel was $32.6 \pm 0.9°$ ($N = 34$). The tunnels were much longer than the chambers (Figure 1b, Table 1). The tunnel dimensions did not differ between the cross sections (width: $Z = 0.909$, $p = 0.363$; height: $Z = 1.727$, $p = 0.084$); therefore, the width and height of the tunnel at two cross sections were averaged. The heights of the tunnels was lower than those of the chambers, whereas width was similar (Table 1).

3.2. Influence of nest structure on nest environments

The real and artificial nests had similar thermal environments in terms of mean, maximum, minimum, and fluctuating range of temperatures (Figure 2a; mean: $F_{2,10} = 0.509$, $p = 0.616$; maximum: $F_{2,10} = 1.232$, $p = 0.333$; minimum: $F_{2,10} = 1.616$, $p = 0.247$; range: $F_{2,10} = 2.642$, $p = 0.120$). The relative humidity was significantly higher and more stable in the real nests and artificial burrow nests than in the artificial non-burrow nests

Table 1.

Comparison of burrow dimensions between the tunnel and chamber in the nest of *Phrynocephalus przewalskii*.

	N	Tunnel	Chamber
Length (cm)	34	25.3 ± 0.8	3.4 ± 0.1
Width (cm)	34	2.3 ± 0.04	2.3 ± 0.1
Height (cm)	34	1.2 ± 0.03	3.1 ± 0.1

(Figure 2b; mean: $F_{2,10} = 42.675$, $p < 0.001$; maximum: $F_{2,10} = 15.718$, $p = 0.001$; minimum: $F_{2,10} = 49.755$, $p < 0.001$; range: $F_{2,10} = 27.467$, $p < 0.001$). Neither soil moisture and oxygen concentration differ among
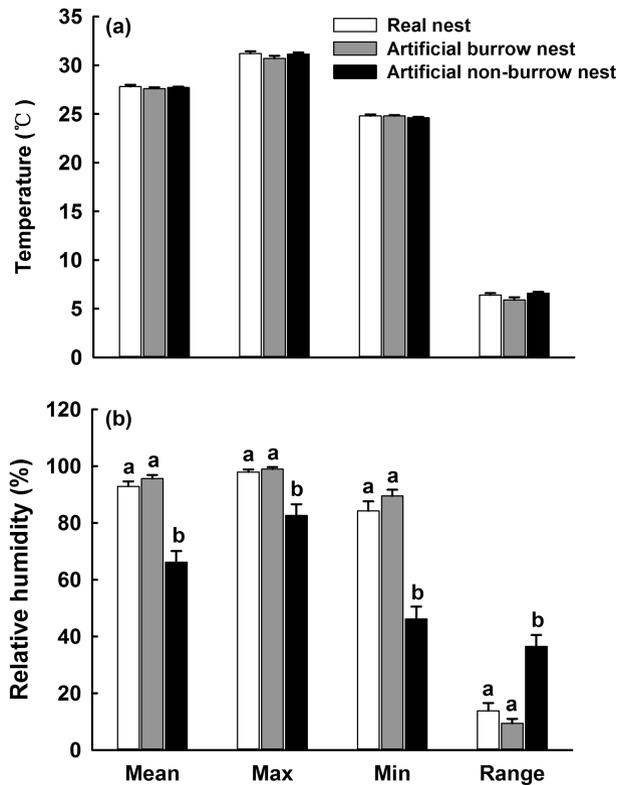

Figure 2. (a) Thermal properties and (b) relative humidity of real nests, artificial burrow nests and non-burrow nests. Data are expressed as mean ± SE. Means with different alphabets above their error bars were statistically different.
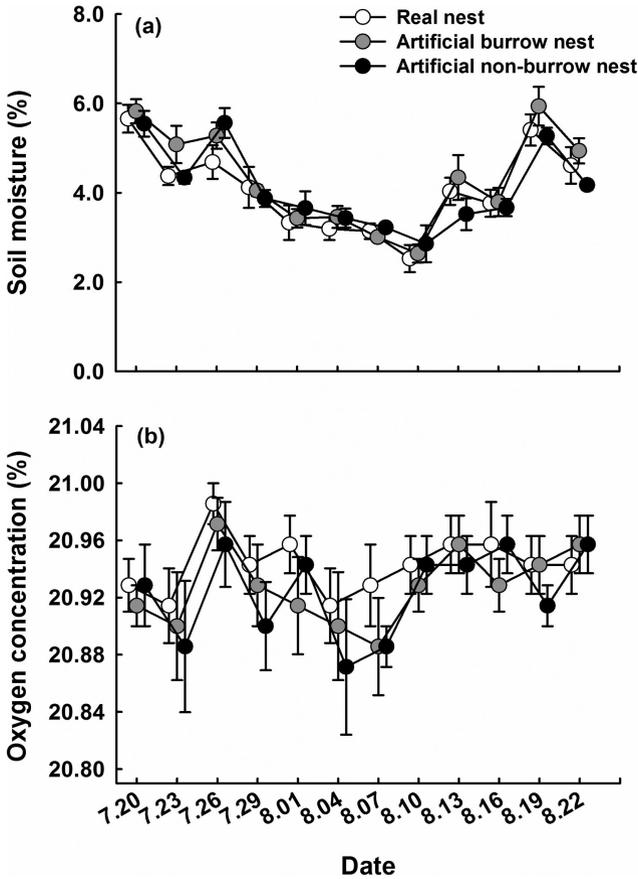
Figure 3. (a) Soil moisture and (b) oxygen concentration of real nests, artificial burrow nests and non-burrow nests during incubation. Data are expressed as mean ± SE.

different types of nests (soil moisture: $F_{2,197} = 2.462$, $p = 0.088$; oxygen concentration: $F_{2,232} = 2.057$, $p = 0.130$), despite fluctuating through the incubation period (soil moisture: $F_{11,197} = 35.139$, $p < 0.001$, Figure 3a; oxygen concentration: $F_{11,232} = 2.843$, $p = 0.002$, Figure 3b).

3.3. Influence of nest structure on embryonic development and hatchling traits

Overall, hatching success was 72.2% (26/36). Eggs from the burrow nests had greater survival than those from the non-burrow nests (88.9% (16/18) *vs.* 55.6% (10/18), one-tailed Fisher's exact test, $p = 0.030$, Figure 4a). In
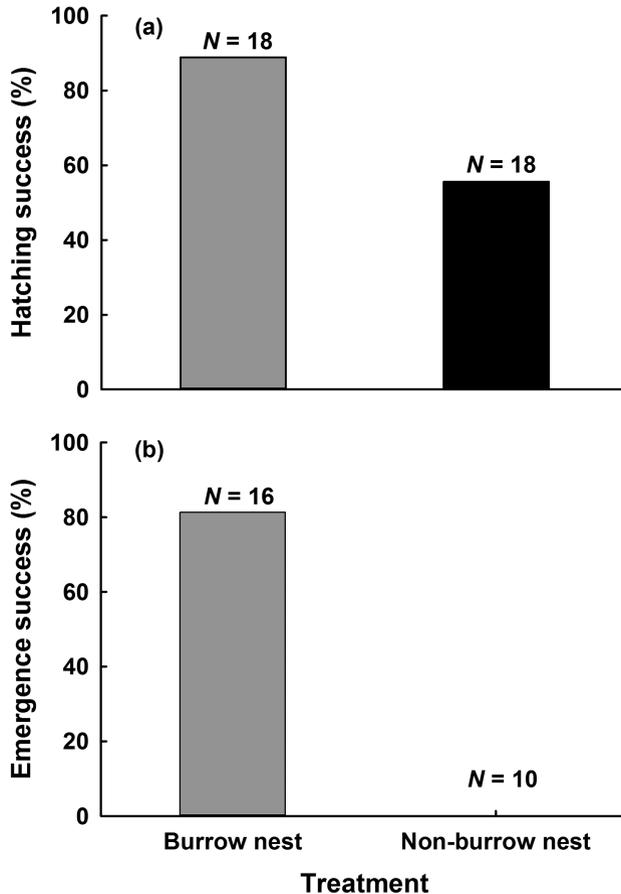
Figure 4. (a) The effect of nest structure on egg survival (hatching success), (b) and hatchling emergence success. Sample sizes are indicated on the figure.

addition, emergence success was higher for hatchlings from the burrow nests than for the non-burrow nests (one-tailed Fisher's exact test, $p < 0.001$). The majority of hatchlings (13/16) from the burrow nests reached the surface, whereas none emerged from the non-burrow nests (Figure 4b).

The initial egg mass was similar between burrow (0.536 ± 0.016, $N = 18$) and non-burrow nests (0.530 ± 0.019, $N = 18$) ($F_{1,34} = 0.065$, $p = 0.800$). Incubation duration was not related to egg mass ($F_{1,21} = 0.243$, $p = 0.627$) and did not differ between the two types of artificial nests (Table 2). Two hatchlings from the burrow nests and one from the non-burrow nests escaped before their morphology could be measured and therefore were not

Table 2.
Incubation duration and morphology of *Phrynocephalus przewalskii* hatchlings from artificial burrow and non-burrow nests.

Variable	Burrow nests	Non-burrow nests	Statistical significance
Incubation duration (day)	39.8 ± 1.0	41.2 ± 1.2	$F_{1,24} = 0.75, \, p = 0.40$
Snout-vent length (mm)	26.1 ± 0.3	25.6 ± 0.4	$F_{1,20} = 0.19, \, p = 0.67$
Tail length (mm)	35.6 ± 0.7	34.3 ± 0.8	$F_{1,21} = 1.24, \, p = 0.28$
Body mass (g)	0.63 ± 0.03	0.59 ± 0.03	$F_{1,20} = 0.43, \, p = 0.52$

One-way ANOVA was used to compare the between-nest difference in incubation duration and tail length. One-way ANCOVA was used to compare the between-nest difference in snout-vent length and body mass, with initial egg mass as the covariate.

included in the morphological analysis. Hatchling SVL and BM were positively related to egg mass (SVL: $r^2 = 0.448$, $F_{1,21} = 18.870$, $p < 0.001$; Body mass: $r^2 = 0.297$, $F_{1,21} = 10.298$, $p = 0.004$), but hatchling TL was not ($F_{1,21} = 3.488$, $p = 0.076$). After the effect of egg mass on hatching size and mass had been removed statistically, hatchlings from the burrow and non-burrow nests did not differ in body size and mass (Table 2).

4. Discussion

Our study demonstrates that a lizard from the desert steppe can construct unique burrows to improve reproductive success. This adds new knowledge to nest construction behaviour in reptiles, which is scarcer than in other taxa, such as birds and fish (Barber, 2013; Heenan, 2013; Mainwaring et al., 2014). This study also highlights the importance of nest structure in determining reproductive success, something that has traditionally been ignored, in addition to the importance of maternal nest-site selection on embryonic development and offspring fitness in oviparous reptiles (Mitchell et al., 2013, 2015).

The nest of *P. przewalskii* was rather deep relative to its small body size — nest depth was three times the mean SVL (15 cm *vs.* 5 cm). This is in contrast to the depth of most reptile nests, that are approximately the same, or smaller than their body length (Thompson et al., 1996; Wilson, 1998; Warner & Shine, 2008; Angilletta et al., 2009). For example, only 15% (4/26) of lizard species build a nest with a depth of over 2 times their SVL, and such deep nests have not been reported in snakes ($N = 8$), turtles ($N = 37$) or crocodiles ($N = 6$) (Figure 5). This kind of unusual deep nest is likely to improve egg survival by avoiding the extremely hot and dry surface soil of the
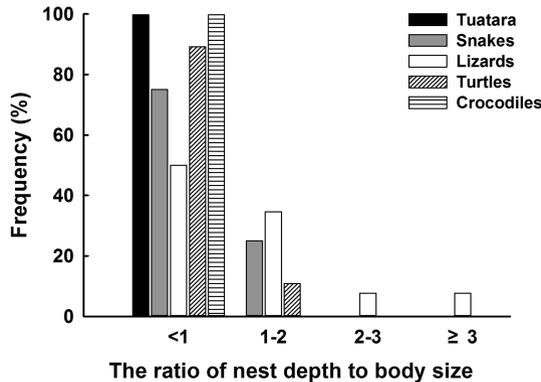
Figure 5. The distribution of the ratio of nest depth relative to adult body size in reptiles (SVL for tuataras ($N = 1$), snakes ($N = 8$), lizards ($N = 26$) and crocodiles ($N = 6$) and carapace length for turtles ($N = 37$)). Data of nest depth and adult body size were collected from the literature. Mean value or median of nest depth and body size was used in the analysis when multiple data sources or data range were available.

desert. In our study site, soil temperature and water content at a 5-cm depth could reach 40°C for nearly four hours per day, and decreased only 0.5% in the summer; such high temperatures are lethal for *P. przewalskii* embryos. Soil temperature and water content at a 15-cm depth was rarely higher than 34°C and 2% lower in the summer, providing a suitable environment for *P. przewalskii* embryos (Zeng et al., 2016; Li et al., unpublished observations). Similarly, a monitor lizard (*Varanus panoptes*) in Australian deserts lays eggs in a very deep nest (average 230 cm depth, 6 times its body length) to avoid extreme heat and dry conditions (Doody et al., 2015).

The structure of *P. przewalskii* burrow nests has also been described in tuatara (*Sphenodon punctatus*) and Allen cays rock iguana (*Cyclura cychlura*), which also consist of a sloping tunnel and an expanded chamber (Thompson et al., 1996; Iverson et al., 2004). The structure of such nests is simpler than that of large lizards nests, such as the interconnected underground tunnels of the green iguana (*Iguana iguana*) (Rand & Dugan, 1983) and the deep helical burrow of the monitor lizard (*V. panoptes*) (Doody et al., 2015), but more complex than those of some lizards, which bury their eggs in shallow underground nests covered with soil or sand (Warner & Shine, 2008; Angilletta et al., 2009).

Our results support the hypothesis that the burrow nests enhance egg survival. The higher hatching success of eggs from the burrow nests was not

because of temperature difference, since thermal properties were independent of nest structure. No impact of the nest structure on nest temperature has also been reported in turtle nests (Burger, 1976; Matsuzawa et al., 2002), and in underground burrows of banner-tailed kangaroo rats (Kay & Whitford, 1978). By contrast, nest structure does optimize the nest thermal environment in birds (Rockweit et al., 2012; Heenan, 2013). Some birds use insulated materials to prevent heat loss in above-ground nests (Reid et al., 2002; McGowan et al., 2004), and others use organic materials to generate heat in underground nests (Seymour & Ackerman, 1980).

A possible explanation to the enhanced egg survival in burrow nests might be the hydric and respiratory environment of the nests, which can significantly affect hatching success of reptiles (Reedy et al., 2013; Cheng et al., 2015). In *P. przewalskii*, burrow nests had higher and more stable atmosphere humidity than non-burrow nests, although soil moisture did not differ between the two types of nests (Figures 2, 3). Similarly, the humidity in rodent burrows has been found to be 80–90%, independent of substrate moisture (Šumbera et al., 2004). The high and stable humidity in burrow nests would be more suitable for egg incubation, and hence improve the hatching success of eggs in this species, because high and stable humidity is important for reptilian embryonic development (Deeming, 2004; Lourdais et al., 2007). Reptiles prefer to lay eggs at relatively moist sites (Socci et al., 2005; Reedy et al., 2013). Our study further indicated that lizards construct burrow nests to create a suitable hydric environment for successful development of eggs. This highlights the importance of nest humidity in maternal nest-site selection, which was less studied in previous studies in reptiles.

The oxygen concentration (19–20%) in the nests of arthropods, lizards and rodents (Anderson & Ultsch, 1987; Christian & Lawrence, 1991; Šumbera et al., 2004), which was independent of burrow depth and entrance status (open or sealed) (Roper et al., 2001), is only slightly lower than normal oxygen concentration in the atmosphere (21%). The oxygen concentration in *P. przewalskii* nests is not an exception, and the oxygen concentration in burrow nests is similar to that in non-burrow nests (Figure 3). Nonetheless, previous studies showed that air chambers in turtle nests facilitated gas exchange and therefore improved embryonic development (Ackerman, 1980; Cheng et al., 2015), because hatching success declined when the air chambers were filled with sand (Simon, 1975). Such difference may be due to the lower egg oxygen consumption in *P. przewalskii*, whose clutch mass (1–2 g) is tiny

compared to the huge clutch mass (more than 2 kg) in sea turtles (Ackerman, 1980).

Our results also indicate that burrows can enhance hatchling emergence success. The low emergence success of hatchlings in non-burrow nests is likely to be related to physical limitations, because nests were possibly too deep (mean nest depth 15 cm *vs.* mean hatchling SVL 2.5 cm) for hatchlings to dig out of. In addition, the wide head and stubby trunk of this species make it more difficult to squeeze through the sand, and therefore hamper their digging capabilities. A similar phenomenon has been reported in sea turtles; hatchlings failed to emerge from nests where the chambers caved in (Mortimer, 1990).

In conclusion, the nest structure is simple but vital for egg survival and hatchling emergence in *P. przewalskii*. Our manipulation experiments provide direct evidence that maternal nest construction behaviour could determine parental reproductive success, and highlight the ecological importance of reptilian nests in embryonic development. Therefore, the ecological consequences of nest structure (e.g., the long-term effects on offspring fitness) merit further attention in future studies. More generally, our study suggests that oviparous reptiles, like lizards, provide ideal models to study the adaptive significance of maternal nest construction behaviour, because reptile development is extremely sensitive to nest environment (Deeming, 2004; Du & Shine, 2015).

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