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Thermal Effects on Embryogenesis and Hatchlings of the Grass Lizard *Takydromus stejnegeri* (Squamata: Lacertidae) and Implications of Their Potential for Limiting Its Altitudinal Distribution in Taiwan

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Yi-Huei Chen, Shu-Ping Huang, Mu-Hsuan Chang, and Ming-Chung Tu (2010) Thermal effects on embryogenesis and hatchlings of the grass lizard *Takydromus stejnegeri* (Squamata: Lacertidae) and implications of their potential for limiting its altitudinal distribution in Taiwan. *Zoological Studies* **49**(3): 374-380. Among the life stages of oviparous reptiles, embryo and hatchling are particularly susceptible to temperatures. Since temperature decreases with altitude, the upper altitudinal limit of some lowland species could be related to the temperature requirement for embryogenesis. The endemic grass lizard *Takydromus stejnegeri* is widely distributed at altitudes below 1000 m in Taiwan. In this study, we examined the thermal effects on embryogenesis of this species and evaluated its potential influence on current altitudinal distribution of *T. stejnegeri*. We measured some indices of embryogenesis and hatchling after incubation at 5 constant temperatures (15, 20, 25, 30, and 33°C). The results revealed that (1) hatching success increased with temperatures from 15°C to 20°C, reached 100% at 25°C and 30°C, and then declined at 33°C; and (2) the incubation duration increased with decreasing temperatures. Based on polynomial regression analysis, we predicted the development of embryos would cease at 16°C. Comparing the monthly average temperature at 3 different altitudes, we found that the *T. stejnegeri* would have an incubation success <100 % at areas higher than 1000 m (monthly average soil temperature < 25°C), and its eggs would not have enough time to hatch in the areas above 1600 m. We assumed that the temperature requirement for embryogenesis could be a limiting factor for the current altitudinal distribution of *T. stejnegeri*. http://zoolstud.sinica.edu.tw/Journals/49.3/374.pdf

Key words: Thermal effect, Altitudinal distribution, *Takydromus stejnegeri*, Incubation temperature.

The distribution pattern of a species is normally related to environmental factors such as temperature, moisture, salinity, and oxygen partial pressure (Brown and Lomolino 1998). The environmental temperature is particularly important for the distribution patterns of ectothermic animals since it greatly influences body temperature and consequently physiological properties (Bennett 1980, Angilletta 2001) such as the metabolic rate, activity levels, and locomotor performance (Huey 1982, Lourdais et al. 2004). Because the thermal environment changes with altitude, it is reasonable to speculate that temperature plays an important role in the altitudinal distribution of reptiles.

Temperature may limit the distribution by causing illness or death of animals. Thermal tolerance can be an important index for the altitudinal distribution of species, and this has mostly been tested in adult individuals (Huang et al. 2006 2007, Huang and Tu 2008a b). However, natural selection presumably acts on every stage of an animal's life cycle (Packard et al. 1977). The thermal environment can influence stages in the life history other than the adult stage, such as the embryo and hatchling stages. Particularly in oviparous animals, the embryo stage is supposed to be a key stage because embryos are passively exposed to external environments for a long period

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of time owing to their inability to escape from the nest site. An organism adjusts its physiological conditions to changes in environmental physical factors, and this phenomenon may be particularly important for developing embryos (Lindström 1999). The success of a natural population often depends as much upon adaptations of the embryos as upon those of adults (Packard et al. 1977). Moreover, especially in oviparous animals, for which a long period of development occurs outside the mother's body, the incubation environments to which the eggs are exposed can deeply influence the phenotypes of the offspring (Lindström 1999, Deeming 2004). For ectotherms, temperature is an important factor that affects the developmental rate and final body size (Atkinson 1994). Other investigations also revealed that the phenotypes of reptilian hatchlings differ with variations in the incubation temperature such as the average temperature (Shine and Harlow 1996, Flatt et al. 2001).

Parker and Andrews (2007) stated that the incubation temperature is an important limiting factor for the latitudinal distribution of oviparous reptiles. Moreover, if an oviparous reptile cannot exist at a high latitudinal area due to low incubation temperatures, then its distribution boundary could be predicted by the thermal tolerance of the embryo and developmental rates at different temperatures. Similar to the latitudinal distribution, the altitudinal distribution may also be strongly influenced by temperature during incubation. In Taiwan, the endemic grass lizard *Takydromus stejnegeri* is widely distributed at altitudes below 1000 m (Lue et al. 1999). According to a previous study (Huang and Tu 2008b), the cold tolerance of adults is not a crucial biological constraint on its current altitudinal distribution. In this study, we attempted to clarify whether the thermal effects on embryogenesis provide a biotic constraint on its current altitudinal distribution. In order to examine this hypothesis, we examined and compared embryogenesis and the phenotypes of hatchlings of *T. stejnegeri* under 5 constant-temperature treatments (15, 20, 25, 30, and 33°C).

MATERIALS AND METHODS

Animals and egg collection

From Aug. to Sept. 2003, we collected female *T. stejnegeri* in Taipei. Pregnant females, which were confirmed by palpating their bellies, were individually maintained in plastic boxes (21 (length) × 12 (width) × 12 cm (height)) with moistened moss as a substrate. They were fed crickets (approximately 5 mm long in body size) every day. Mealworms and water were provided ad libitum. The environmental temperature was set to 25°C with a 12:12 h L:D photoperiod (lights on at 06:00-18:00).

In a preliminary test, we found that females always slept at night and never laid eggs from 20:00 to 06:00. Based on this observation, we collected eggs at 11:00, 16:00, and 21:00 to ensure that eggs were collected within 5 h of being laid.

Egg incubation and data collection

We used 5 constant incubation temperatures in our study: 15, 20, 25, 30, and 33°C. In order to prevent exposure to an extremely high temperature, eggs were incubated at 33°C instead of 35°C.

Since water loss in the early developmental phase may cause high mortality (Lin and Ji 1998), females are inclined to lay eggs when the moisture of the soil matrix just exceeds a certain threshold (Warner and Andrews 2002). When eggs are incubated in wet vermiculite as the matrix, moisture does not significantly influence hatching success (Lin and Ji 1998, Flatt et al. 2001, Shine 2005), incubation duration (Shine 2005), or phenotypes of the hatchlings (Lin and Ji 1998). If the soil matrix provides enough water for embryogenesis, the effect of moisture on development is insignificant. Consequently, we decided to use a mixture, which vermiculite: water of 1: 2, as the incubation matrix for our eggs. We buried the eggs halfway into the matrix and maintained the same orientation as when they were originally laid. In order to maintain the same moisture content, water was added to the matrix every 2 d.

Only 1 egg from each female was assigned to a randomly chosen temperature. The time at which we collected each egg was recorded, and the initial egg mass was measured. Mass and snout-vent length (SVL) of the females were measured after they laid eggs.

The egg mass was recorded every 2 d during the incubation period. Approximately 1 wk before the expected hatching date (based on a preliminary test), we checked for hatchlings at 09:00 and 21:00 daily. The hatching time, mass of the hatchlings, SVL, and tail length were measured. The residual egg mass was dried to a constant weight. The residual dry egg mass was recorded to compare the utilization of the yolk at different incubation temperatures. During the hatching process, if the egg presented serious dehydration, mold, or a brown surface, it was considered dead. Hatching success was calculated after all eggs had hatched.

Environmental temperature

Soil temperatures are more relevant to embryogenesis than air temperatures. Unfortunately, soil temperatures are not normally available. A mid-altitude (at 1000 m) observatory of the Central Weather Bureau at Sun Moon Lake (120°53'60"E, 23°52'59"N) recorded both the daily average soil temperature at a depth of 5 cm and the daily air temperature. The daily average soil temperature at a depth of 5 cm was 1.5°C higher than the daily average air temperature (2003-2007). Therefore, we added 1.5°C to the air temperature recorded at the altitudes of 1600 m (Shenmu Village, 120°50'05"E, 23°32'10"N) and 1400 m (Fenqi Lake, 120°41'28"E, 23°29'45"N) to obtain the approximate daily average soil temperature at these 2 altitudes.

Data analysis

We used an analysis of variance (ANOVA) to test whether the initial egg mass at the 5 different temperatures were randomly distributed. To calculate the hatching duration, we defined the median of the 3 times (09:00, 13:30, and 18:30) at which the eggs were collected as the time at which the eggs were laid. The median of the 2 times (03:00 and 15:00) at which the hatchlings were collected was used as the time at which the eggs hatched. The developmental rate was defined as the reciprocal of the number of days until hatching. A polynomial regression analysis was used to examine the relationship between the incubation temperature and developmental rate.

The body size of the offspring might be an important element of reptilian fitness. For example, extreme high or low temperatures that result in hatchlings that are too small may weaken their competitive ability (Ferguson and Fox 1984). For this reason, we also examined the hatchling mass, SVL, and tail length at different incubation temperatures by analysis of covariance (ANCOVA). The hatchling mass, SVL, tail length, and residual dry egg mass were tested by ANCOVA with the initial egg mass as the covariance in order to evaluate the effect of incubation temperatures. Tukey's honest significant difference (HSD) test was used to test significant differences among the 5 temperatures. We used JMP software (vers. 6.0.0; SAS institute, Cary, NC, USA) to perform all statistical analyses described above. Data are presented as the mean ± standard deviation (SD) unless otherwise indicated.

RESULTS

We collected 27 female lizards, but the information on 2 individuals was lost. The SVL of females ranged 48.3-62.4 (mean ± SD: 55.8 ± 3.3) mm (*n* = 25), and the mass ranged 2.00-4.18 (mean ± SD: 3.02 ± 0.53) g. From 11 Aug. to 25 Sept. 2003, 1, 2, and 3 clutches of eggs were produced by 6, 14, and 5 females respectively. We found 6, 17, and 28 clutches of eggs at 11:00, 16:00, and 21:00, respectively.

The initial egg masses at the 5 temperature treatments did not significantly differ (*F* ratio = 1.87, *p* = 0.15). The average egg mass was 0.212 ± 0.021 (range, 0.179-0.269) g. Egg masses visually differed among the temperature treatments, with the lowest incubation temperature resulting in the lowest increasing rate of egg mass (Fig. 1). Hatching success increased as the temperature increased and peaked at 25 and 30°C and then declined at 33°C. The incubation duration was shortened when the temperature increased (Table 1).

We used a polynomial regression method to examine the relationship between the incubation temperature and developmental rate (Table 2). A

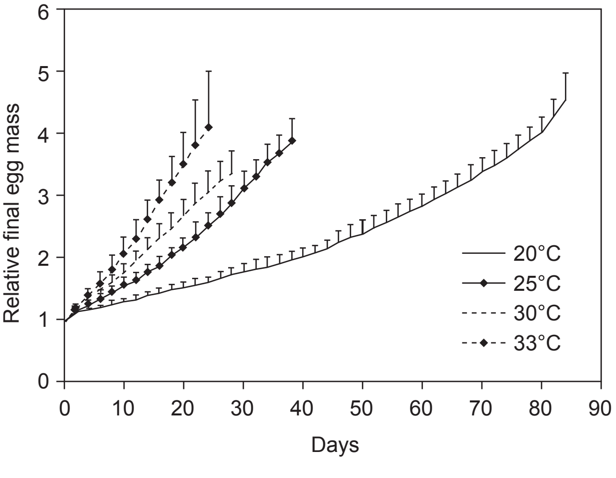

Fig. 1. Relationship between relative final egg mass and the incubation periods at 20, 25, 30, and 33°C. On the y-axis, the relative final egg mass was calculated as (egg mass $_{\text{ith day}}$)/ (egg mass $_{\text{the 1st day}}$). Values are shown as the mean + SD.

partial F-test was used to check which model fit the best. The best-fit model of developmental rate and temperature was as follows: Rate (1/d) = -0.000051 × temperature^2 + 0.004758 × temperature - 0.06311 (adjusted R^2 = 0.994). We extrapolated this formula and found that the development of *T. stejnegeri* embryos should cease when the average incubation temperature is < 16.0°C (Fig. 2).

Table 3 shows the values for the hatchling mass, SVL, tail length, and residual dry egg mass for each treatment. The initial egg mass significantly influenced the hatchling mass, SVL, and residual dry egg mass (hatchling mass: F ratio = 8.20, $p < 0.05$; SVL: F ratio = 5.79, $p < 0.05$; residual dry egg mass: F ratio = 24.02, $p < 0.01$) but not the tail length of hatchlings (F ratio = 4.48, $p = 0.053$). The incubation temperature did not significantly influence the hatchling mass, SVL, tail length, or residual dry egg mass (hatchling mass: F ratio = 0.86, $p = 0.85$; SVL: F ratio = 1.16, $p = 0.35$; tail length: F ratio = 1.32, $p = 0.309$; residual dry egg mass: F ratio = 0.08, $p = 0.970$).

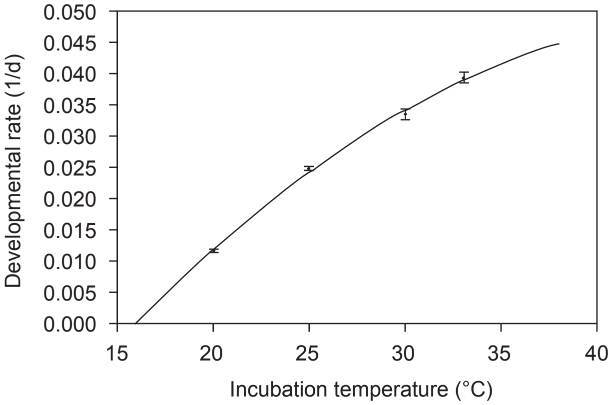

Fig. 2. Relationship between the developmental rate and incubation temperature of *Takydromus stejnegeri*. This formula was built using a polynomial regression method. The developmental rate has a quadratic declining trend as the incubation temperature decreases. The developmental rate is expected to reach 0% when the incubation temperature is about 16°C.

DISCUSSION

Hatchlings of *T. stejnegeri* that hatched at 20, 25, 30, and 33°C showed no significant differences in mass, SVL, tail length, or residual dry egg mass. Most previous investigators reported that the incubation temperature influences phenotypes, especially the body size of reptilian hatchlings (Atkinson 1994, Shine and Harlow 1996, Braña and Ji 2000, Ji and Zhang 2001, Shine 2004, Willingham 2005). Studies on *Takydromus septentrionalis* also revealed that higher temperatures led to smaller hatchling size and more residual yolk mass than did lower temperatures (Lin and Ji 1998, Du and Ji 2006). Nevertheless, some studies showed that the temperature influences hatchling mass because of differences in the water content. The incubation temperature did not have a significant effect on the dry masses of eggs (Pan and Ji 2001, Du and Ji 2006). Although it seems a common viewpoint that hatchlings incubated at lower temperatures are larger in size and the yolk is more completely used (Packard and Packard 1988), some studies showed that the incubation temperature significantly influenced only some or none of the indices of body size of reptilian hatchlings (Flatt et al. 2001, Pan and Ji 2001, Hare et al. 2004, Parker and Andrews 2007). Consequently, thermal effects on the phenotypes of hatchlings may vary among different species, and they do not exhibit a consistent pattern.

Hatching success reaches a plateau in a certain temperature range and declines thereafter in many lizards (Lin et al. 2005). This suitable temperature range is defined as the optimal incubation temperature (Packard et al. 1977, Webb et al. 1983). When the incubation temperature is set to the outside of the suitable range, the probability of abnormalities and mortality rises.

Table 1. Hatching success, incubation duration, and egg mass at different incubation temperatures

Temperature (°C)	15	20	25	30	33
Hatching success (%)	0	71.4	100	100	80
Incubation duration (d)[a]		86.0 ± 2.1	40.4 ± 0.6	30.0 ± 0.7	25.5 ± 0.6
Initial egg mass (g)[b]	0.207 ± 0.009	0.214 ± 0.007	0.196 ± 0.009	0.215 ± 0.009	0.229 ± 0.009
n	5	7	5	5	5

[a]Mean ± SD. [b]Least squares mean ± S.E.

We defined the optimal temperature as the temperatures at which incubation success reaches 100%. In our study, the optimal incubation temperatures ranged 25-30°C. When observing the estimated monthly average soil temperature at altitudes above 1000 m in Taiwan (Fig. 3), we found that it was always < 25°C at these altitudes. It seems clear that *T. stejnegeri* is only widely distributed below 1000 m because the temperature above an altitude of 1000 m is not suitable for its embryogenesis.

According to the results for the developmental rate, we can further predict the altitudinal limit of *T. stejnegeri*. The developmental rate was correlated to the incubation temperature, and results showed that embryos required a longer time to hatch at lower incubation temperatures. The time required for incubation at 20°C was 84.7 d and that at 17°C was 317.2 d (Table 4). On the other hand, the

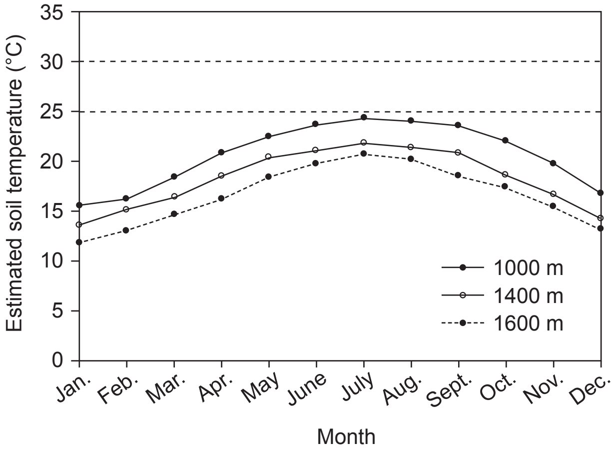

Fig. 3. The estimated monthly average soil temperature at altitudes of 1000, 1400, and 1600 m. The 2 horizontal dotted lines indicate the optimal incubation temperature of *Takydromus stejnegeri* in our study. The monthly average soil temperatures at altitudes above 1000 m are < 25°C.

Table 2. Result of polynomial regression analysis on the embryonic development rate

Parameters	Full model		Model 1	
	β	*p*	β	*p*
Intercept	-0.02708	***	-0.02926	***
Incubation temperature (Temp.)	0.00206	***	0.00209	***
(Temp. -26.6842) × (Temp. -26.6842)	-0.00005	**		
Degree of freedom	2		1	
F ratio	1488.87	***	1291.84	***
Adjusted R^2	0.994		0.986	

**$p < 0.01$, ***$p < 0.0001$.

Table 3. Body mass, snout-vent length (SVL), tail length, and dry egg mass of hatchlings in 4 different temperature treatments. All data are shown as the least squares mean ± S.E.

Temp.	*n*	Mass (g)	SVL (mm)	Tail length (mm)	Residual dry egg mass (mg)
20°C	5	0.277 ± 0.01	22.76 ± 0.66	46.08 ± 3.02	15.52 ± 0.72
25°C	5	0.281 ± 0.02	23.65 ± 0.76	53.76 ± 3.50	15.88 ± 0.84
30°C	5	0.271 ± 0.01	23.89 ± 0.66	45.79 ± 3.02	15.48 ± 0.72
33°C	4	0.259 ± 0.02	21.95 ± 0.88	43.80 ± 4.05	15.20 ± 0.97

Table 4. Estimated available time (d) at 3 altitudes that exceed the incubation duration of *Takydromus stejnegeri* at various suboptimal temperatures

Temperature (°C)		16	17	18	19	20
Incubation duration (d)		∞	317.2	162.9	110.8	84.7
Excess available time (d)	1000 m	-∞	-1.2	114.1	138.2	144.3
	1400 m	-∞	-86.2	41.1	66.2	44.3
	1600 m	-∞	-122.2	-6.9	-4.8	-9.7

temperature decreases as the altitude increases; thus, the time available for embryos to hatch would decrease as the altitude increases. When the available time exceeds the time required for incubation, the eggs might still have a chance to hatch. When the available time is less than the time required for incubation, the eggs will not be able to hatch, and the value of excess available time becomes negative. For example, at an incubation temperature of 20°C, the excess available time at an altitude of 1000 m was 144.3 d, and this decreased to 44.3 d at an altitude of 1400 m. This value became negative at 1600 m, which indicates that there are not enough days for embryos to hatch at this altitude. At an altitude of 1600 m, the number of excess available days at various suboptimal temperatures was negative (Table 4). This suggests that even at suboptimal temperatures, there are not enough days for *T. stejnegeri* to hatch at altitudes exceeding 1600 m.

In addition to temperature, many other factors can interact with one another to affect the distribution limits of a species (Hutchinson 1957, Packard and Packard 1988). According to the findings of this study, the wide distribution of *T. stejnegeri* below 1000 m can be explained solely on the basis of thermal effects, and we predict that it would be impossible to establish a population of *T. stejnegeri* above 1600 m. With more accurate data on soil temperatures at various locations and more detailed information on embryogenesis of various ectotherms, it would be possible to draw a boundary line of altitudinal distribution for each species. This could also lead to a more definite conclusion that the thermal effect on embryogenesis is a limiting factor in the altitudinal distribution of oviparous reptiles.

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