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Incubation temperature fluctuation does not affect incubation length and hatchling phenotype in the Chinese skink *Plestiodon chinensis*

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ABSTRACT

Studies examining the effects of incubation temperature fluctuation on the phenotype of hatchling reptiles have shown species variation. To examine whether incubation temperature fluctuation has a key role in influencing the phenotype of hatchling Chinese skinks (*Plestiodon chinensis*), we incubated eggs produced by 20 females under five thermal regimes (treatments). Eggs in three treatments were incubated in three incubators, one set constant at 27 °C and two ramp-programmed at 27 ± 3 °C and 27 ± 5 °C on a cycle of 12 h (+) and 12 h (−). The remaining eggs were incubated in two chambers: one inside a room where temperatures varied from 23.0 to 31.1 °C, with a mean of 27.0 °C; the other outside the room where temperatures varied from 20.2 to 35.3 °C, with a mean of 26.1 °C. We found that: (1) for eggs at a given embryonic stage at ovipositon, the mean rather than the variance of incubation temperatures determined the length of incubation; (2) most (egg mass, embryonic stage at oviposition, incubation length and all examined hatchling traits except tail length and locomotor performance) of the examined variables were affected by clutch; and (3) body mass was the only hatchling trait that differed among the five treatments, but the differences were tiny. These findings suggest that incubation temperature fluctuation has no direct role in influencing incubation length and hatchling phenotype in *P. chinensis*.

1. Introduction

For the embryos of ectotherms, temperature affects both physiological processes during development and the subsequent phenotypic attributes of individuals at birth or hatching. In reptiles, for example, temperatures experienced by eggs during incubation affect not only the rate of embryonic development but hatchling traits that are sensitive to temperature, including body size, morphology, survival, post-hatching growth, immune response, locomotor performance, behavior, cognition, and even sex in species with temperature-dependent sex determination (TSD) (Packard and Packard, 1988; Deeming, 2004; Valenzuela, 2004; Booth, 2006; Amiel et al., 2014). Most studies have been conducted in the laboratory at constant temperatures. In recent years we have seen a growing number of studies where eggs are incubated under controlled, fluctuating temperatures in the laboratory (e.g., Neuwald and Valenzuela, 2011; Warner and Shine, 2011; Lin et al., 2008; Li et al., 2013a,b). The results are broadly applicable to natural incubation conditions (Shine et al., 1997; Nelson et al., 2006; Wang et al., 2013), but the temperature effects demonstrated in the laboratory often do not reflect what can be found under natural conditions because eggs in natural nests are subjected to fluctuating temperatures on a daily and seasonal basis (Cagle et al., 1993; Overall, 1994; Ackerman and Lott, 2004; Birchard, 2004; Booth, 2006). For example, reptilian embryos generally develop successfully across a 5–8 °C range of constant temperatures but can develop across a much broader range of temperatures in natural nests provided that exposure of eggs to extreme temperatures is intermittent (Birchard, 2004; Li et al., 2013a).

One of the main obstacles to examining the temperature effects in nature has been the difficulty in locating nesting sites. Given this difficulty, alternative research protocols have to be designed. One approach consists of mimicking natural cyclicity of temperature to some extent in laboratory settings (Andrews and Rose, 1994; Andrews et al., 2000; Ashmore and Janzen, 2003; Andrewartha et al., 2010; Du and Shine, 2010; Li et al., 2013a,b). Alternatively, eggs are incubated in "outdoor incubators" where they can be exposed to naturally fluctuating temperatures (Castilla and Swallow, 1996; Du and Ji, 2006; Lu et al., 2009; Li et al., 2012; Löwenborg et al., 2012). Studies of incubating reptilian eggs under thermal conditions mimicking natural cyclicity or in "outdoor

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incubators" have shown that fluctuating temperatures are important in some situations, but their importance varies among species and even populations, and may affect some traits but not others (Neuwald and Valenzuela, 2011; Warner and Shine, 2011; Li et al., 2013a,b; Refsnider, 2013). Where fluctuating temperatures influence embryogenesis differently than constant temperatures, that effect can result from the temperature fluctuation *per se*, or the fact that temperature fluctuation results in exposure of eggs to extreme temperatures likely causing major phenotypic modifications (Li et al., 2013a,b). Thus, to address the question of whether temperature fluctuation has a role in influencing embryogenesis, we need to study the temperature effects on a species-specific and trait-specific basis.

Here, we examined the effects of incubation temperature fluctuation on incubation length and hatchling phenotype in the Chinese skink *Plestiodon* (formerly *Eumeces*) *chinensis* by incubating eggs at one constant and four fluctuating temperatures (see below for details). *P. chinensis* is a medium-sized (up to 134 mm snout–vent length, SVL; Lin and Ji, 2000), ground-dwelling, oviparous scincid lizard that ranges from central and southern China to Vietnam (Zhao and Adler, 1993). We chose this skink for study because: (1) its life history and reproductive biology are well known; (2) clutch size (8–38 eggs) is relatively large, allowing subdivision of single clutches into several experimental treatments; and (3) the temperature range (24–32 °C) within which incubation success is high (>82%) and the temperature range (26–30 °C) within which the phenotypes of hatchlings are optimal are well known (Lin and Ji, 2000; Ji and Zhang, 2001; Ji et al., 2002; Du et al., 2005; Lu et al., 2012, 2014). Our objectives were (1) to examine whether fluctuating temperatures influence embryogenesis differently than constant temperatures, and (2) to relate incubation length and hatchling phenotype to incubation temperature fluctuation.

2. Materials and methods

2.1. Animal collection and husbandry

We collected 20 gravid females (89–118 mm SVL) in mid-May 2007 from Lishui (28°27′N, 119°55′E; ~70 m elevation), Zhejiang, East China. Females were transported to our laboratory in Hangzhou, where five were housed in each of four outdoor enclosures (length × width × height: $1.5 \times 1.5 \times 0.6$ m^3) with a substrate of moist soil (~150 mm depth) covered with grass. Females had the opportunity to regulate body temperature through selective exploitation of natural thermal flux. Mealworm larvae (*Tenebrio molitor*), house crickets (*Achetus domestica*) and water enriched with vitamins and minerals were provided daily. Females laid a single clutch of 9–25 eggs between 27 May and 23 June. During this time period, we checked the enclosures at least thrice daily for freshly-laid eggs. Body mass and SVL were recorded for each post-oviposition female. All females were released at their sites of capture in late June soon after the last female laid eggs.

2.2. Egg collection and incubation

Eggs were collected and weighed less than 3 h post-laying, thereby minimizing water gain or loss between the egg and the substrate (Ji and Zhang, 2001). Fertilized eggs could be easily identified by visual inspection due to the presence of a reddish embryonic disk. Of the 302 eggs collected, 268 (~89%) were fertilized and could be incubated. One fertilized egg from each clutch was dissected for identification of Dufaure and Hubert's (1961) developmental stage at oviposition, and the remaining eggs were individually placed into 50 ml covered plastic jars filled with moist vermiculite (water potential ≈ −12 kPa). Eggs from the same clutch were assigned as equally as possible among the five temperature treatments.

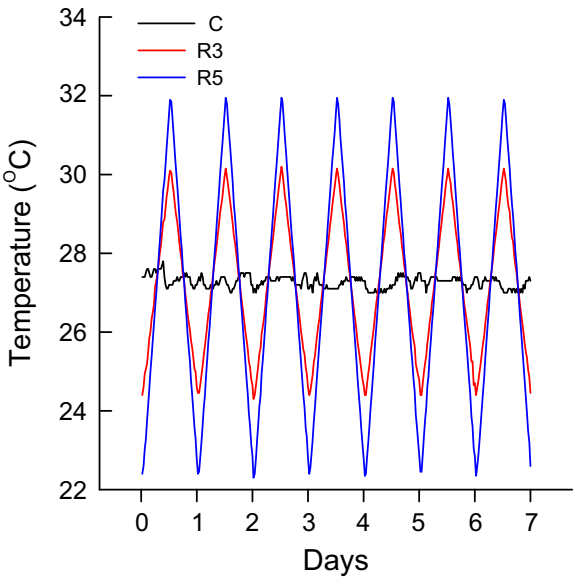

Fig. 1. Representative temperature profiles for a 7 d period in C, R3 and R5 temperature treatments. C treatment: eggs in an incubator set at 27 °C (black line); R3 and R5 treatments: eggs in two incubators ramp-programmed at 27 ± 3 °C (red line) and 27 ± 5 °C (blue line), respectively. Temperatures in the R3 and R5 treatments changed on a cycle of 12 h (+) and 12 h (−). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Eggs in three treatments were incubated in three Binder KB incubators (Binder, Germany): one was set at 27 °C (C treatment); two were ramp-programmed at 27 ± 3 °C (R3 treatment) and 27 ± 5 °C (R5 treatment), respectively. Temperatures in the R3 and R5 treatments both changed on a cycle of 12 h (+) and 12 h (−), and were monitored with Tinytalk temperature loggers (Gemini Pty, Australia) programmed to record temperature at 30 min intervals on seven consecutive days (Fig. 1). We rotated jars at 2 d intervals to minimize the possible influence of thermal gradients inside the incubator. Substrate water potential was adjusted at 5 d intervals by individually weighing jars, and water was added to compensate for evaporative losses and water taken up by the egg.

Eggs in the remaining two treatments were placed in two $400 \times 300 \times 200$ mm^3 chambers: one (F1 treatment) was placed in a room, and the other (F2 treatment) was placed in a bush-covered backyard outside the room. These two treatments were supposed to address the influence of less predictable and more extreme temperatures. Thermal fluctuations in each chamber were monitored with a Tinytalk temperature logger inserted amongst the eggs to record temperatures at 30 min intervals throughout the experimental period. One-way analysis of variance (ANOVA) showed that the thermal mean ($F_{1,\ 38}=7.61$, $P<0.01$) and minimum ($F_{1,\ 38}=28.87$, $P<0.0001$) were greater in the F1 treatment, whereas the thermal variance ($F_{1,\ 38}=73.87$, $P<0.0001$) and maximum ($F_{1,\ 38}=423.37$, $P<0.0001$) were greater in the F2 treatment (Table 1).

Incubation length was defined as the time interval between oviposition and piping. Within a half day of hatching, hatchlings were collected, weighed, and measured firstly for locomotor performance and then for morphological traits.