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Evolution of Reptilian Viviparity: A Test of the Maternal Manipulation Hypothesis in a Temperate Snake, *Gloydius brevicaudus* (Viperidae)

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We kept 48 gravid short-tailed pit vipers (*Gloydius brevicaudus*) under four laboratory thermal conditions during gestation and collected 10 females from the field soon before they gave birth to test whether Shine's (1995) maternal manipulation hypothesis applies to temperate reptiles. Females thermoregulated more precisely but did not shift their selected body temperatures during pregnancy, with females at high body temperatures giving birth early in the breeding season. The lowest (22°C) and highest (32°C) temperature treatments increased maternal mortality and resulted in production of offspring with smaller body dimensions. More deformed offspring were produced at 32°C, and more poorly performing offspring were produced at 22°C. In the field, air temperatures lower than 22°C and higher than 32°C accounted for about 9% and 33% of total temperature readings, respectively. However, offspring produced by field-caught females did not differ from those produced by laboratory-kept females with body temperatures optimal for embryonic development in nearly all traits examined. This suggests that in nature, gravid females avoid exposure of their embryos to temperature extremes through thermoregulation. Our study validates the key prediction of the maternal manipulation hypothesis that maternal thermoregulation should enhance fitness-related offspring traits, and demonstrates that viviparity evolves in temperate reptiles because internal development shields offspring from temperature extremes.

Key words: Viperidae, *Gloydius brevicaudus*, viviparity, gestation temperature, morphology, performance, growth, temperate climate

INTRODUCTION

Viviparity is a reproductive mode that evolves from oviparity through gradual increases in the length of egg retention and intrauterine development (Andrews and Mathies, 2000). This reproductive mode characterizes all mammals except monotremes, and it has also had more than 150 independent origins within fishes (Dulvy and Reynolds, 1997; Goodwin et al., 2002; Reynolds et al., 2002), amphibians (Duellman and Trueb, 1986; Wilkinson and Nussabaum, 1998; García-París et al., 2003), and reptiles (Blackburn, 2000; Shine, 2005) in terrestrial, marine, or freshwater environments. Viviparity offers pervasive benefits by lowering embryonic mortality, accelerating embryonic development, optimizing the offspring phenotype, and obviating the need for females to find suitable egg-laying sites. Nonetheless, viviparity entails several costs such as increased maternal mortality, decreased fecundity or reproductive output, and reduced genetic diversity among offspring as a consequence of reduced matings or clutches, thereby limiting the occurrence of this reproductive mode (Tinkle and Gibbons, 1977; Shine, 2005).

Squamate reptiles (lizards, snakes and amphisbaenians) provide a very useful model system for studying the evolutionary transition from oviparity to viviparity and the adaptive significance of this transition, because they exhibit a wide reproductive diversity well beyond a simple oviparity/viviparity dichotomy (Shine, 1983, 2005; Heulin et al., 2002). Unlike other reptilian taxa in which females deposit when embryos are in the gastrula (turtles and tuataras) or the neurula (crocodilians) stage, squamate reptiles exhibit nearly the entire gamut of possible embryonic stages at oviposition, with embryonic stages at oviposition grouping around a mode of Dufaure and Hubert's (1961) stage 30 (lizards) or Zehr's (1962) stage 27 (snakes) in most species (Shine, 1983; Xavier and Gavaud, 1986; Andrews and Mathies, 2000). Moreover, approximately 20% of squamate reptiles are viviparous, and this reproductive mode has evolved far more often in this group of animals (> 100 lineages) than in all other non-mammalian vertebrates combined (Shine, 2005).

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The selective forces that led to the evolution of viviparity within squamate reptiles have received extensive study for many decades. Most hypotheses postulate that viviparity has evolved in squamate reptiles for thermal reasons; that is, thermal differentials between the uterus and the nest resulting from maternal thermoregulation are the key to the evolution of viviparity (Shine, 1995, 2004, 2005; Blackburn, 2000). Shine's (1995) maternal manipulation hypothesis predicts that gravid females in thermally challenging environments should adjust thermoregulation to provide more suitable or predictable temperatures for their developing offspring, and that phenotypic traits determined by maternal thermoregulation should enhance offspring fitness. Recent studies of two warm-climate (the Northern Death Adder, *Acanthophis praelongus*, Webb et al., 2006; the Many-lined Sun Skink, *Mabuya multifasciata*, Ji et al., 2007) and one cold-climate (the Gobi Racerunner, *Eremias prezwalskii*, Li et al., 2009) viviparous species support the main predictions from Shine's (1995) hypothesis. The maternal manipulation hypothesis should apply to all situations or species where gravid females are able to enhance offspring (and thus, their own) fitness by maintaining more stable but not always higher body temperatures for developing embryos than those available in external nests (Webb et al., 2006), yet it has never been tested in any temperate reptile. Here, we describe a study where gravid short-tailed pit vipers (*Gloydius brevicaudus*) were kept under five thermal conditions during gestation to examine the effects of maternal temperature on developmental rates and offspring phenotypes.

MATERIAL AND METHODS

Species studied

The short-tailed pit viper is a medium-sized (up to 620 mm snout-vent length, SVL), viviparous, highly venomous snake that has an exclusively temperate distribution ranging from eastern China (southward to Fujian and Guangdong, westward to Guizhou and Sichuan, northward to Shaanxi and Gansu, and northeastward to Liaoning) to Korea (Zhao, 1998). Females often ovulate in June or slightly later, and give birth in late summer between August and September (Hu et al., 1966; Zhao, 1998). The areas occupied by *G. brevicaudus* are characterized by large daily thermal fluctuations, and ambient temperatures in summer months (from June to September) are often too high or low for embryonic development of many squamate reptiles sympatric with the snake (Lu et al., 2009a and included references). Therefore, *G. brevicaudus* is well suited to testing the hypothesis that viviparity evolves in temperate reptiles because internal development shields offspring from temperature extremes.

Animal collection and treatment

We conducted five treatments, four of them in the laboratory. Forty-eight laboratory-kept adult females (> 425 mm SVL) were collected in late June 2008 from a population in Xiaoshan (30°11'N, 120°17'E), Zhejiang, eastern China. Females were brought to our laboratory at Hangzhou Normal University (~15 km away from the collection site), where they were palpated to confirm that all were ready to ovulate or had just ovulated, before being assigned to one of the four laboratory conditions (thermal treatments). Six females were housed together in one 1 m × 0.5 m × 0.9 m (length × width × height) glass cage, which had a substrate consisting of 0.1 m of moist soil, clay tiles, and grass. Food (Chinese loaches [*Paramisgumus dabryanus*]) and water enriched with vitamins and minerals were provided ad libitum. Placing multiple snakes in a single cage might generate problems associated with social interactions; however, in no cage did we observe a social hierarchy, competition over food, or competition for the best basking spots in the thermoregulatory treatment.

Females (N = 36) in three of the four laboratory treatments, with 12 individuals in each, were maintained in three 3 m × 4 m AAPS (artificial atmospheric phenomena simulator) rooms, where they could maintain body temperatures at 22, 27, and 32 (± 0.5)°C, respectively. Fluorescent tubes, which automatically switched on at 07:00 h (Beijing time), were on a 12:12-h light:dark cycle. Body (cloacal) temperature was taken for each female by using a WMZ-3 digital thermometer (Shanghai Medical Instrument, China) to verify that the mean body temperature was controlled at the anticipated level. The lowest (22°C) and highest (32°C) temperatures chosen were approximately 3°C above or below the range (~25–29°C) of body temperatures selected by gravid females but within the range of air temperatures the snakes encountered in summer months (Fig. 1).

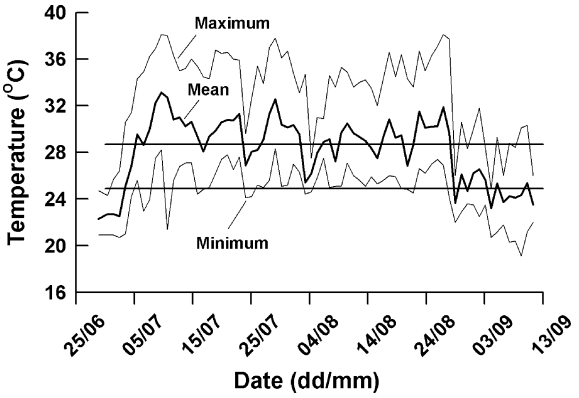

Fig. 1. Temporal variation in daily minimal, mean, and maximal air temperatures gravid females encountered in nature. Daily mean temperatures ranged from 22.3 to 33.1°C, with an average of 28.2 ± 0.3°C; daily minimal temperatures ranged from 19.1 to 28.3°C, with an average of 24.6 ± 0.3°C; daily maximal temperatures ranged from 24.3 to 38.1°C, with an average of 31.1 ± 0.4°C. Body temperatures selected by gravid females in a cost-free laboratory thermal gradient were never higher than 28.7°C (the upper horizontal line) or lower than 24.9°C (the lower horizontal line).

Females (N = 12) in the fourth laboratory treatment (hereafter, the TR [thermoregulation] treatment) were maintained in an AAPS room where the temperature was controlled at 20 ± 0.5°C. A 100-W ceramic heater suspended above one end of each cage created a thermal gradient ranging from the room temperature to ~55°C (5 cm above the cage floor) for the whole experiment period, so that females in the cage had ample opportunity to regulate body temperature within their voluntary range.

Females (N = 10) in the fifth treatment (hereafter, the F [field] treatment) were collected from the same population in early September, with all of them giving birth in the laboratory within one week after capture. Three Tinytalk dataloggers (Gemini Pty, Australia) programmed to record the temperature every 1 h were placed 1 m above the ground surface and 200 m apart at the site where snakes were collected, so that temporal variation in air temperature could be automatically recorded. The maximum magnitude of daily thermal fluctuations, and the mean, lowest, and highest air temperatures between late June and mid-September at the site where snakes were collected were 16.6, 28.2, 19.1, and 38.1°C, respectively (Fig. 1).

Female reproduction

As soon as the first female gave birth, the cages were checked

at least twice daily for newborns so that neonates could be always collected, measured, and weighed within a few hours after parturition. Females giving birth during the same period were isolated from each other in 0.4 m × 0.4 m × 0.4 m wire cages, so that offspring could be allocated accurately to the mother. Snout-vent length and body mass were measured for each postpartum female. Newborns were individually measured to the nearest millimeter for SVL and tail length with a ruler, and weighed to the nearest milligram on a Mettler top loading balance. We determined the sex of offspring (including deformed young and stillborns) by pressing on both sides of the ventral tail base to record the presence or absence of hemipenes, and those with everted hemipenes were recorded as males. To measure variation in offspring size, we calculated the CV (coefficient of variation) for neonate mass for each litter by dividing the standard deviation by the mean, and then multiplying by 100.

Thermal preference

An independent sample of eight gravid females, seven non-gravid females, and seven adult males (> 490 mm SVL) collected from the same population in early August 2008 was used to examine whether females shifted their thermal preference during pregnancy. This experiment was conducted in 1 m × 0.5 m × 0.9 m plastic cages placed in a room at about 20°C. A 100-W ceramic heater suspended above one end of each cage created a thermal gradient ranging from 20 to 55°C for the whole experimental period. Seven to eight snakes, with gravid females, non-gravid females, and adult males randomly mixed, were introduced into the thermal gradient at the cool side. Body temperatures were taken for each snake at four time points (04:00, 10:00, 16:00, and 22:00 h) on two consecutive days using the same thermometer mentioned above.

Offspring phenotypes

We evaluated locomotor performance of offspring by measuring their swimming stamina and speed inside a bath (1.5 m × 0.15 × 0.45 m) filled with water to a height of 0.25 m, which allowed vertical filming with a Panasonic NV-DS77 digital video camera (Panasonic, Japan). The water temperature was maintained at 28°C via a water bath heater fixed to a metal stand. A WMZ-3 digital thermometer confirmed that the water temperature did not vary more than 0.5°C during trials. We individually placed neonates into the bath and then repeatedly tapped on the tail with a paintbrush to encourage them to swim. The time it took for each neonate to swim until exhaustion was considered to be its swimming stamina. The tapes were examined with a computer using MGI VideoWave III software (MGI Software, Canada) to determine the speed in the fastest 0.25 m interval.

Following performance measurements, two neonates (one female and one male) were randomly selected from each litter to collect additional data (head length, head width, and the number of ventral scales). These neonates were then killed by freezing to –15°C, and were separated into carcass, residual yolk, and fat bodies, which were dried in an oven (60°C) to constant mass. The remaining neonates were moved into one of fifteen 0.6 m × 0.5 m × 0.4 m plastic cages placed in a room where temperatures varied from 22°C to 28°C. Small pieces of Chinese loach were provided in excess and spread throughout the cage, so that the neonates had free access to food. The neonates were weighed again on Day 90 to evaluate growth in the first 90-day period.

Statistical analyses

We used the Statistica software package (version 5.0 for PC) to analyze data. We tested data for normality using the Kolmogorov-Smirnov test and for homogeneity of variances using Bartlett's test (univariate level) and/or Box's M test (multivariate level). Percentage data were arcsine transformed prior to parametric analyses. We used the paired-sample *t*-test, the *G* test, linear regression analysis, partial correlation analysis, one- and two-way analysis of variance (ANOVA), repeated measures ANOVA, one-way analysis of covariance (ANCOVA), multivariate analysis of covariance (MANCOVA), multivariate analysis of variance (MANOVA), and Tukey's post-hoc

Table 1. Descriptive statistics for female reproductive traits in *G. brevicaudus*.

	Thermal treatments					The results of one-way ANCOVA or ANOVA
	F	TR	22	27	32	
N	10	9	5	12	6	
Snout-vent length (mm)	498.5 ± 14.3 428–558	544.3 ± 13.1 480–605	516.8 ± 14.9 475–558	524.2 ± 10.7 473–570	561.7 ± 15.5 480–570	$F_{4, 37} = 1.65$, $P = 0.182$
Postpartum body mass (g)	64.5 ± 5.4 36–95	78.2 ± 7.6 42–123	71.4 ± 10.2 46–96	80.9 ± 7.1 47–125	64.3 ± 9.1 44–94	$F_{4, 36} = 1.21$, $P = 0.324$
Litter size	9.2 ± 1.1 5–16	11.1 ± 1.4 6–18	9.4 ± 0.5 8–11	10.8 ± 1.0 6–19	11.5 ± 0.8 9–15	$F_{4, 36} = 0.41$, $P = 0.798$
Litter mass (g)	32.8 ± 4.9 17.8–64.4	43.4 ± 5.1 17.0–59.4	31.1 ± 4.9 18.5–45.1	41.4 ± 3.4 23.2–65.9	37.9 ± 4.5 29.4–59.4	$F_{4, 36} = 0.78$, $P = 0.544$
Neonate mass (g)	3.50 ± 0.12 2.71–4.02	3.94 ± 0.23 2.84–5.06	3.25 ± 0.38 2.32–4.51	3.85 ± 0.09 3.47–4.50	3.25 ± 0.15 2.90–3.96	$F_{4, 36} = 2.39$, $P = 0.067$
CV of offspring size	10.9^{ab} ± 2.0 3.9–23.4	8.7^{ab} ± 2.1 3.2–20.0	10.1^{ab} ± 1.2 7.0–14.0	5.1^{b} ± 0.6 3.0–8.8	17.3^{a} ± 4.6 7.2–34.9	$F_{4, 37} = 4.05$, $P < 0.008$
Proportion of female offspring (%)	37.6 ± 5.2 14.3–62.5	50.2 ± 4.4 25.0–75.0	45.9 ± 6.1 22.2–57.1	54.8 ± 5.2 16.7–84.6	56.0 ± 6.4 40.0–77.8	$F_{4, 37} = 2.05$, $P = 0.107$
Proportion of deformed offspring (%)	1.7^{b} ± 1.7 0–16.7	7.9^{b} ± 3.7 0–27.1	0^{b}	0.6^{b} ± 0.6 0–7.1	71.7^{a} ± 15.7 0–100	$F_{4, 37} = 26.62$, $P < 0.0001$
Proportion of stillborns (%)	8.3^{bc} ± 4.5 0–33.3	9.6^{bc} ± 4.7 0–33.3	29.3^{b} ± 7.8 0–45.5	2.1^{c} ± 2.1 0–25.0	68.3^{a} ± 12.6 33.3–100	$F_{4, 37} = 19.06$, $P < 0.0001$

Values are expressed as mean values ± SE and ranges. SVL was the covariate in all ANCOVA (for postpartum body mass, neonate mass, litter size, and litter mass) models. Mean values with different superscripts differ significantly (Tukey's post-hoc test, $\alpha = 0.05$; a > b > c). CV, coefficient of variation; F, females collected from the field soon before parturition; TR, females maintained in the laboratory thermal gradient; 22, 27, 32, females maintained in controlled temperature rooms where the temperature was set at 22, 27, and 32°C, respectively.

test to analyze the corresponding data. Homogeneity of slopes was checked prior to testing for differences in adjusted means. Throughout this paper, values are presented as mean values ± SE, with the significance level set at $\alpha = 0.05$.

RESULTS

Female reproduction

Of the 48 laboratory-kept females, 32 gave birth and the remaining 16 died at various stages of pregnancy (Table 1). Maternal mortality differed among the four laboratory treatments ($G = 14.67$, $df = 3$, $P < 0.005$), and was much higher in the 22°C and 32°C treatments. Birth date, expressed as the number of days since the first day of August, differed among the five treatments (one-way ANOVA; $F_{4, 37} = 69.51$, $P < 0.0001$), with females at high body temperatures giving birth early in the breeding season (Fig. 2).

Females under the five thermal conditions did not differ from each other in SVL, postpartum body mass, litter size, litter mass, or neonate mass (Table 1). Pooling data from different treatments, we found that that litter size ($r^2 = 0.45$, $F_{1, 40} = 32.21$, $P < 0.0001$) (Fig. 3A), litter mass ($r^2 = 0.57$, $F_{1, 40} = 53.69$, $P < 0.0001$) (Fig. 3B), and neonate mass ($r^2 = 0.20$, $F_{1, 40} = 9.83$, $P < 0.004$) (Fig. 3C) were all positively correlated with female SVL. The proportion of female offspring did not differ among the five treatments; embryonic mortality and offspring-size variability were both highest in the 32°C treatment and lowest in the 27°C treatment; the proportion of deformed offspring with either trunk or tail malformations was much higher in the 32°C treatment than in the other four treatments (Table 1).

Thermal preference

Body temperatures measured at each of the four time points did not differ between the two consecutive days in gravid females, non-gravid females, or adult males (paired-sample *t*-test; all $P > 0.110$). Pooling data from the two days (Table 2), we found that neither reproductive condition (repeated measures ANOVA; $F_{2, 19} = 1.32$, $P = 0.289$) nor time point (repeated measures ANOVA; $F_{3, 57} = 2.49$, $P = 0.069$) affected body temperature, and that the reproductive condition × time step interaction was not a significant source of variation in body temperature (repeated measures ANOVA; $F_{6, 57} = 1.50$, $P = 0.196$). The standard deviation (SD) in body temperature differed among gravid females, non-gravid females, and adult males (ANOVA; $F_{3, 57} = 5.62$, $P = 0.012$), and so did coefficient of variation (CV) of body temperature (ANOVA; $F_{3, 57} = 6.67$, $P < 0.007$). The SD and

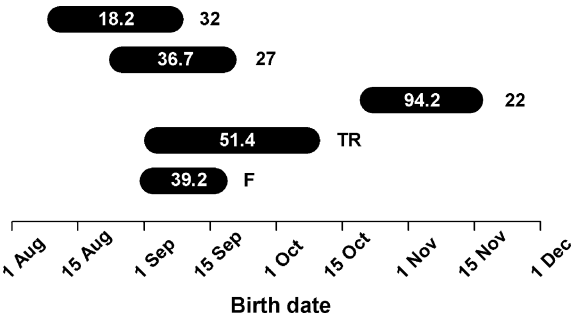

Fig. 2. Birth dates of offspring produced by females maintained under different thermal conditions. Numbers in the horizontal bars indicate mean days since the first day of August. F, females collected from the field soon before parturition; TR, females maintained in the laboratory thermal gradient; 22, 27, 32, females maintained in controlled temperature rooms where the temperature was set at 22, 27, and 32°C, respectively.

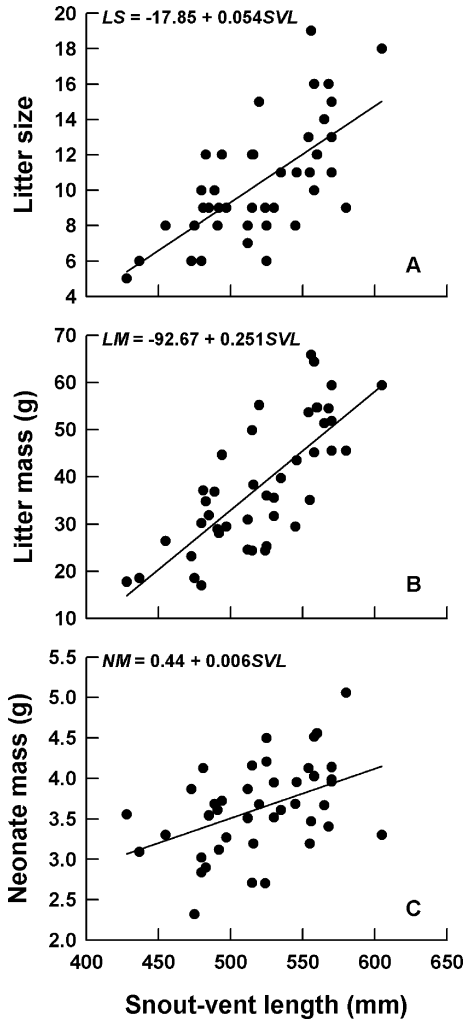

Fig. 3. Linear regressions of litter size, litter mass, and neonate mass on female SVL. The regression equations are given in the figure.

Table 2. Descriptive statistics for body temperatures (°C) selected by gravid females, non-gravid females, and adult males in the laboratory thermal gradient.

	Gravid females	Nongravid females	Adult males
N	8	7	7
Body temperature	26.9 ± 0.5	26.5 ± 1.1	25.1 ± 0.9
	24.9–28.7	22.7–31.1	22.7–28.4
SD of body temperature	2.9 ± 0.3	4.3 ± 0.5	3.8 ± 0.4
	0.6–3.8	2.7–5.5	1.8–4.9
CV of body temperature	9.2 ± 1.1	16.3 ± 2.0	15.0 ± 1.3
	2.2–13.1	8.6–22.2	7.9–18.4

CV for body temperature were both greatest in non-gravid females and smallest in gravid females, with adult males in between (Table 2).

Offspring phenotype

Table 3 shows the size and morphology of offspring from different treatments. The number of ventral scales was not correlated with neonate SVL in any sex × thermal treatment combination (all $P > 0.099$). We therefore used two-way ANOVA to examine the effects of sex, thermal treatment, and their interaction on this trait. Female neonates had more ventral scales than did males ($F_{1, 74} = 83.64$, $P < 0.0001$); the number of ventral scales differed among the five treatments ($F_{4, 74} = 2.77$, $P = 0.034$), with the mean significantly greater in the TR and 27°C treatments than in the 32°C treatment (Tukey's post-hoc test, both $P < 0.05$); the sex × thermal treatment interaction was not a significant source of variation in the trait ($F_{4, 74} = 0.10$, $P = 0.981$). A MANCOVA with neonate SVL as the covariate revealed that tail length, head length, and head width of offspring were affected by both sex (Wilks' lambda = 0.43, $df = 3, 71$, $P < 0.0001$) and thermal treatment (Wilks' lambda = 0.71, $df = 12, 188$, $P = 0.016$) but not by the sex × thermal treatment interaction (Wilks' lambda = 0.90, $df = 12, 188$, $P = 0.814$). Female neonates were smaller in tail length and head length (both $P < 0.013$) than males of the same SVL. Neonates of the same SVL from different treatments differed in head length ($P < 0.006$) but not in tail length or head width (both $P > 0.080$), with head length larger in the TR and 27°C treatments than in the 32°C treatment (both $P < 0.016$).

Within each treatment, female and male neonates did not differ in SVL, body wet mass, body dry mass, carcass dry mass, fatbody dry mass, or residual yolk dry mass (all $P > 0.125$). Blocking data on both sexes by litter, we found that SVL, body wet mass, carcass dry mass, and residual yolk dry mass differed among the five treatments (Table 4). Snout-vent length was smaller in the 22°C and 32°C treatments. SVL-specific body wet mass was greatest in the 32°C treatment and smallest in the F treatment; SVL-specific carcass dry mass was greatest in the 22°C and 32°C treatments and smallest in the F treatment; residual yolk dry mass was greater in the 32°C treatment than in the other four treatments (Table 4). A series of partial correlation analyses revealed that offspring SVL was positively correlated with carcass dry mass ($r = 0.80$, $t = 11.99$, $df = 80$, $P < 0.0001$) but negatively with residual yolk dry mass ($r = -0.48$,

Table 3. Size and morphology of offspring produced by females under different thermal conditions.

	Thermal treatments									
	F		TR		22		27		32	
	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
N	10	10	9	9	5	5	12	12	6	6
Snout-vent length (mm)	181.0 ± 3.5	168.1 ± 4.1	179.8 ± 2.1	175.4 ± 3.9	147.0 ± 0.5	154.3 ± 3.7	176.3 ± 1.5	176.0 ± 1.6	161.8 ± 3.3	150.0 ± 6.6
	161–197	141–182	167–187	151–186	146–149	146–165	170–187	163–185	150–172	126–176
Tail length (mm)	26.2 ± 0.5	28.5 ± 0.9	27.5 ± 0.7	30.7 ± 1.1	21.6 ± 0.6	26.0 ± 1.1	27.2 ± 0.7	30.6 ± 0.6	22.5 ± 0.6	23.7 ± 1.3
	23.7–29.7	23.4–32.1	24.6–31.6	26.2–35.8	20.1–23.5	22.1–28.5	23.7–32.5	27.4–33.7	20.7–24.5	20.5–29.0
Head length (mm)	12.8 ± 0.1	12.6 ± 0.2	12.9 ± 0.1	13.1 ± 0.1	11.6 ± 0.2	12.0 ± 0.2	12.9 ± 0.1	12.9 ± 0.1	11.9 ± 0.2	11.8 ± 0.4
	11.7–13.2	11.5–13.2	12.2–13.4	12.2–13.6	10.8–12.2	11.5–12.6	12.5–13.3	12.4–13.5	11.3–12.4	10.3–12.6
Head width (mm)	6.5 ± 0.08	6.3 ± 0.11	6.7 ± 0.11	6.7 ± 0.07	5.7 ± 0.09	6.2 ± 0.1	6.5 ± 0.05	6.7 ± 0.1	6.0 ± 0.1	6.0 ± 0.3
	6.1–7.0	5.5–6.7	6.2–7.1	6.4–7.2	5.4–6.0	6.0–6.7	6.3–7.0	6.3–7.6	5.6–6.4	4.7–6.6
Ventral scales	142.6 ± 0.7	138.8 ± 0.5	143.1 ± 0.6	139.0 ± 0.6	142.6 ± 0.5	138.2 ± 0.6	142.9 ± 0.5	138.6 ± 0.6	140.7 ± 1.0	137.0 ± 1.2
	140–146	136–141	140–145	136–141	141–144	137–140	139–146	135–142	138–145	133–141

Table 4. Body mass and composition of offspring produced by females under different thermal conditions.

	Thermal treatments					The results of one-way ANCOVA or ANOVA
	F	TR	22°C	27°C	32°C	
N	10	9	5	12	6	
Snout-vent length (mm)	$174.6^{a} \pm 3.3$	$177.6^{a} \pm 2.7$	$150.7^{b} \pm 2.1$	$176.1^{a} \pm 1.3$	$155.9^{b} \pm 3.8$	$F_{4, 37} = 17.18$, $P < 0.0001$
	151.0–186.4	162.1–186.5	145.8–157.0	167.4–185.9	147.4–173.9	
Body wet mass (g)	$3.45^{b} \pm 0.16$	$3.93^{ab} \pm 0.17$	$3.01^{ab} \pm 0.20$	$3.76^{ab} \pm 0.07$	$3.38^{a} \pm 0.25$	$F_{4, 36} = 2.88$, $P = 0.036$
	2.34–3.97	3.28–4.57	2.44–3.68	3.35–4.23	2.66–4.38	
Body dry mass (mg)	754.9 ± 38.3	822.6 ± 38.2	680.0 ± 40.4	806.4 ± 17.4	745.7 ± 44.9	$F_{4, 36} = 2.38$, $P = 0.070$
	573.0–935.5	663.5–992.3	565.0–816.5	708.8–897.9	605.0–875.5	
Carcass dry mass (mg)	$653.1^{b} \pm 29.5$	$713.3^{ab} \pm 30.6$	$571.3^{a} \pm 32.5$	$704.7^{ab} \pm 12.4$	$631.9^{a} \pm 40.6$	$F_{4, 36} = 3.27$, $P = 0.022$
	494.0–749.0	585.5–844.0	472.0–677.0	625.0–780.3	513.5–760.0	
Fatbody dry mass (mg)	76.3 ± 7.9	91.7 ± 14.0	78.8 ± 6.4	82.5 ± 7.1	60.3 ± 6.7	$F_{4, 37} = 1.22$, $P = 0.321$
	40.0–125.0	40.0–150.0	60.0–95.0	40.0–135.0	32.5–75.0	
Residual yolk dry mass (mg)	$26.4^{b} \pm 5.7$	$16.4^{b} \pm 5.8$	$31.0^{b} \pm 4.2$	$18.6^{b} \pm 3.2$	$72.3^{a} \pm 18.2$	$F_{4, 37} = 7.68$, $P < 0.0002$
	4.0–65.0	5.0–55.0	23.0–47.0	2.3–37.3	8.5–139.3	

Snout-vent length was the covariate in all ANCOVA models (wet body mass, dry body mass, and dry carcass mass). Mean values with different superscripts differ significantly (Tukey's post-hoc test, $\alpha = 0.05$; a > b).

t = 4.94, df = 80, P < 0.0001); that carcass dry mass was positively correlated with both residual yolk dry mass (r = 0.40, t = 3.90, df = 80, P < 0.0002) and fatbody dry mass (r = 0.36, t = 3.45, df = 80, P < 0.001); and that fatbody dry mass was not correlated with either residual yolk dry mass or offspring SVL (both P > 0.093).

Swimming performances (swimming stamina and speed) of offspring were affected by thermal treatment (MANOVA; Wilks' lambda = 0.04, df = 8, 72, P < 0.0001). Swimming stamina was greatest in the TR treatment and smallest in the 22°C treatment (Fig. 4A); swimming speed was highest in the 27°C treatment and lowest in the F and 22°C treatments (Fig. 4B).

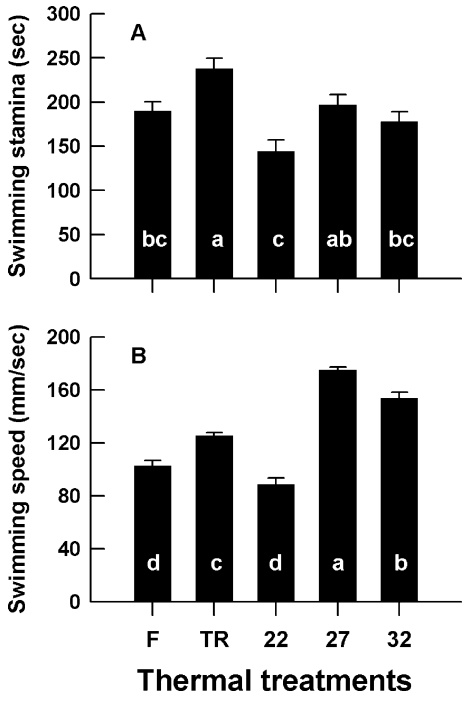

Fig. 4. Mean values (+ SE) for **(A)** swimming stamina and **(B)** swimming speed for offspring from different thermal treatments. Means with different letters differ significantly (Tukey's post-hoc test, α = 0.05; a > b > c > d).

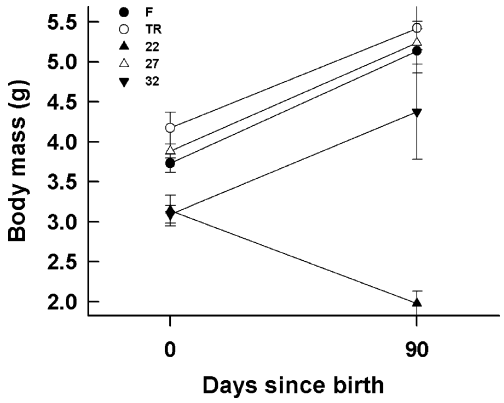

Fig. 5. Mean values (± SE) for the growth of offspring in the first 90-day period.

Offspring from the 22°C treatment lost mass in the first 90-day period, whereas offspring from the other four treatments gained mass (Fig. 5). An ANCOVA with body mass at birth as the covariate showed that the mass gain in this period did not differ among the latter four treatments ($F_{3, 30}$ = 0.09, P = 0.966).

DISCUSSION

Thermal regimes females encountered in the five treatments were so different that their internal (or maternal) temperatures would be certainly different. The finding that maternal temperature does not affect the sexual phenotype of offspring in *G. brevicaudus* adds evidence that the sex of snakes is not determined by embryonic temperature (Janzen and Phillips, 2006). High temperatures result in faster embryonic development and, thus earlier parturition dates in *G. brevicaudus* (Fig. 2). The likely reason why females in the TR treatment gave birth later than those in the constant 27°C treatment was that they selected cooler body temperatures at night, so that their overall mean body temperature would be lower that 27°C. Of the female reproductive traits examined, embryonic mortality, offspring-size variability, and the incidence of deformed offspring were affected by gestation temperature. Of the offspring traits examined, SVL, tail length, head size, SVL-specific body wet mass, SVL-specific carcass dry mass, residual yolk dry mass, the number of ventral scales, swimming performance, and postnatal growth were affected by maternal temperature.

The 22°C and 32°C treatments not only increased maternal mortality but also resulted in the production of offspring with smaller body dimensions (SVL and tail length). Nonetheless, the two treatments differentially affected other thermally sensitive traits, including embryonic mortality and embryonic abnormality, and the residual yolk, swimming speed, and postnatal growth of offspring. The high incidence of stillborns and the increased maternal mortality provide direct evidence that exposure of gravid females to 22°C or 32°C for prolonged periods of time may substantially reduce their reproductive success, simply because maximization of reproductive success should be achieved in females by producing the greatest number of surviving young (Bernardo, 1996; Agrawal et al., 2001; Roff, 2002). Body dimensions at hatching or birth are highly associated with the developmental condition of the body in reptiles, with large offspring often having well developed and thus heavy bodies (Lu et al., 2009a). In oviparous snakes, it is possible for small hatchlings to increase body dimensions by using residual yolk, but this entails additional energetic costs associated with body growth (Ji et al., 1997, 1999). Such an ''ebb and flow'' relationship between the body and residual yolk is similar to that between the embryo and yolk seen during embryogenesis, with the general pattern almost the same among reptiles (Shadrix et al., 1994; Du and Ji, 2001; Lu et al., 2009b). Accordingly, we believe that embryos in the 22°C and 32°C treatments would consume more energy to grow to the same size as embryos in the other three treatments. The highest incidence of deformed offspring in the 32°C treatment provides additional evidence that incubation at high constant temperatures is detrimental to embryos of *G. brevicaudus*. Offspring-size variability was also greatest in the 32°C treatment, but the link through which variation in

offspring size translates into variation in offspring fitness is currently unknown. Variation in offspring size is sometimes related to fitness (better performance and higher survival), but not always (Ji et al., 2003, 2009; Li et al., 2009). No deformed offspring were found in the 22°C treatment, but bad swimming performance and negative rates of postnatal growth (presumably resulting from the low feeding rate) showed that offspring quality was poor in this treatment. These results suggest that constant incubation at either 22°C or 32°C is detrimental to embryos of *G. brevicaudus*.

In a wide variety of squamate reptiles, females shift their thermal preference (upwards or downwards) when they are gravid (Beuchat, 1988; Daut and Andrews, 1993; Mathies and Andrews, 1997; Ji et al., 2006, 2007; Li et al., 2009). Adjustment of thermal preference and thermoregulatory behavior by gravid females could have evolved because of the advantages associated with the thermal optimality that they provide to embryos, and this has been proposed as one cause for the evolution of viviparity in reptiles (Shine, 1995, 2006). Interestingly, however, gravid female of *G. brevicaudus*, as do females of *A. praelongus* (Webb et al., 2006), maintained more stable body temperatures but with a mean value not differing from that for non-gravid females or adult males (Table 2). Not maintaining lower-than-usual body temperatures during pregnancy is quite understandable, because prolonged 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. But why did females in the TR treatment select body temperatures close to 27°C, even though higher body temperatures would have been easily attainable on the thermal gradient? The answer presumably has three aspects. First, the temperature of 27°C is optimal for both gravid females and developing embryos, not only because no females died at 27°C but also because all traits examined were well optimized at this temperature. Second, gravid females benefit less from selecting higher body temperatures in terms of the reduced gestation length, because the length of embryonic development decreases at a decreasing rate as the temperature increases within the range where successful development can take place (Birchard, 2004). In *G. brevicaudus*, for example, the mean gestation length decreased much less dramatically from 27°C to 32°C (18.5 days) than from 22°C to 27°C (57.5 days). Third, gravid females should avoid selecting high body temperatures, because the increased energetic costs associated with increased metabolic rates at high body temperatures may increase reproductive costs (Lin et al., 2008). Offspring in the TR and 27°C treatments were almost the same with respect to the traits examined, and this suggests that body temperatures selected by gravid females in cost-free laboratory thermal gradients are generally optimal for embryonic development.

In the field, air temperatures outside the range of body temperatures selected by gravid females accounted for about 65% (23% lower than 24.9°C; 42% higher than 28.7°C) of total temperature recordings, with about 42% (9% lower than 22°C; 33% higher than 32°C) of the total recorded temperatures detrimental or potentially lethal to embryos (Fig. 1). Interestingly, offspring in the F treatment did not differ from those in the TR or 27°C treatments in nearly all traits examined. The match of offspring phenotypes between these treatments, together with the finding that the mean gestation length did not differ significantly between the F and 27°C treatments ($F_{1, 20} = 1.09$, $P = 0.309$; Fig. 2), suggests that in nature, gravid females might maintain body temperatures with a mean value close to 27°C, which allows them to optimize the phenotype of their offspring. Further data on the body temperatures of free-ranging gravid females are needed to evaluate this hypothesis. Avoidance of even brief exposure of developing embryos to extreme temperatures could have been the selective basis for the evolution of viviparity in reptiles (Beuchat, 1988; Mathies and Andrews, 1997; Shine, 2005). Our study supports this idea, and demonstrates that viviparity buffers offspring from extreme temperatures.

In cost-free laboratory thermal gradients, gravid females of *G. brevicaudus* regulated body temperature more precisely and thus maintained more stable body temperatures than did non-gravid females and adult males, presumably because the range of temperatures optimal for embryonic development is narrow in this species. Enhanced thermoregulatory precision has been reported in gravid females of reptiles in cold (Charland and Gregory, 1990; Peterson et al., 1993; Charland, 1995) and tropical (Webb et al., 2006; Ji et al., 2007) climates, but whether this is a general phenomenon in viviparous reptiles is currently unknown. In *E. prezwalskii*, for example, females do not thermoregulate more precisely when they are gravid (Li et al., 2009).

Our data do not validate the prediction that females should shift their thermal preference during pregnancy to enhance offspring phenotypes, but confirms two predictions of the maternal manipulation hypothesis: females should provide better or more stable thermal conditions for their embryos and, more importantly, maternal thermoregulation should enhance fitness-related offspring traits. Thus, our study supports the maternal manipulation hypothesis, which may explain the selective advantage of viviparity not only in cold- and warm-climate reptiles, but also in temperate reptiles.

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