Influence of Incubation Temperature on Hatching Success, Energy Expenditure for Embryonic Development, and Size and Morphology of Hatchlings in the Oriental Garden Lizard, *Calotes versicolor* (Agamidae)

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ABSTRACT   We incubated eggs of *Calotes versicolor* at four constant temperatures ranging from 24°C to 33°C to assess the effects of incubation temperature on hatching success, embryonic use of energy, and hatchling phenotypes that are likely to affect fitness. All viable eggs increased in mass throughout incubation due to absorption of water, and mass gain during incubation was dependent on initial egg mass and incubation temperature. The average duration of incubation at 24°C, 27°C, 30°C, and 33°C was 82.1 days, 60.5 days, 51.4 days, and 50.3 days, respectively. Incubation temperature affected hatching success, energy expenditure for embryonic development, and several hatchling traits examined, but it did not affect the sex ratio of hatchlings. Hatching success was lowest (3.4%) at 33°C, but a higher incidence of deformed embryos was recorded from eggs incubated at this temperature compared to eggs incubated at lower temperatures. Most of the deformed embryos died at the last stage of incubation. Energy expenditure for embryonic development was, however, higher in eggs incubated at 33°C than those similarly incubated at lower temperatures. A prolonged exposure of eggs of *C. versicolor* at 33°C appears to have an adverse and presumably lethal effect on embryonic development. Hatching success at 24°C was also low (43.3%), but hatchlings incubated at 24°C did not differ in any of the examined traits from those incubated at two intermediate temperatures (27°C and 30°C). Hatchlings incubated at 33°C were smaller (snout-vent length, SVL) than those incubated at lower incubation temperatures and had larger mass residuals (from the regression on SVL) as well as shorter head length, hindlimb length, tympanum diameter, and eye diameter relative to SVL. Hatchlings from 33°C had significantly lower scores on the first axis of a principal component analysis representing mainly SVL-free head size (length and width) and fore- and hindlimb lengths, but they had significantly higher scores on the second axis mainly representing SVL-free wet body mass. Variation in the level of fluctuating asymmetry in eye diameter associated with incubation temperatures was quite high, and it was clearly consistent with the prediction that environmental stress associated with the highest incubation temperatures might produce the highest level of asymmetry. Newly emerged hatchlings exhibited sexual dimorphism in head width, with male hatchlings having larger head width than females. *J. Exp. Zool. 292:649–659, 2002.*   © 2002 Wiley-Liss, Inc.

The most noticeable effects of thermal environments on reptilian eggs incubated in natural nests and under controlled laboratory conditions are hatching success and incubation length (an indicator of the rate of embryonic development). Eggs cannot be incubated successfully at extreme temperatures outside the range yielding viable hatchlings (Vinegar, '74), although brief periods of daily exposure of eggs to temperatures that are lethal to embryos does not necessarily increase embry-

onic mortality (Sexton and Marion, '74; Overall, '94; Shine and Harlow, '96; Andrews et al., '97). When eggs are incubated at viable temperatures, the rate of embryonic development increases with increases in temperature (e.g., Van Damme et al., '92; Overall, '94; Lin and Ji, '98; Ji and Braña, '99; Ji et al., '99b, 2001; Ji and Du, 2001a,b; Ji and Zhang, 2001). High hatching success may substantially enhance reproductive success in both sexes while short incubation length decreases the exposure time of eggs to the effects of adverse biotic or abiotic factors. For species living in the temperate regions, the shortened incubation period means an increased growth period prior to the onset of the first winter. However, incubation temperatures that maximize the rate of embryonic development usually do not maximize hatching success and offspring phenotypes (Van Damme et al., '92; Lin and Ji, '98; Ji and Braña, '99; Ji et al., '99b, 2001; Braña and Ji, 2000; Ji and Du, 2001a,b; Ji and Zhang, 2001). Numerous studies indicate that the thermal conditions experienced during embryogenesis affect a number of morphological, behavioral, and performance-related phenotypes in reptiles (Packard and Packard, '88; Burger, '91, '98; Shine and Harlow, '93, '96; Allstead and Lang, '95; Huey and Berrigan, '96; Shine et al., '97a,b; Ji and Braña, '99; Ji et al., '99b; Braña and Ji, 2000; Ji and Zhang, 2001). Some temperature-induced modifications of phenotypes may have long-term effects on an individual's fitness, and hence they are ecologically important (Webb and Cooper-Preston, '89; Burger, '91, '98; Van Damme et al., '92; Shine, '95; Shine and Harlow, '96; Alberts et al., '97; Lin and Ji, '98; Braña and Ji, 2000; Ji and Zhang, 2001).

In natural nests, eggs of reptiles encounter daily fluctuations in thermal conditions during incubation. The mean and amplitude of the thermal fluctuations can be very different depending on locality, microhabitat, year, and date of oviposition (Overall, '94; Castilla and Swallow, '96; Shine and Harlow, '96; Thompson et al., '96; Shine et al., '97a,b). Therefore, a female's choice of a thermally appropriate nest site is one way through which she selects optimal thermal environments for embryonic development, so that hatching success, incubation length, and offspring phenotypes (and hence offspring fitness) are compromised to a certain extent. However, studying the effects of nest thermal environments on egg incubation in lizards has one technical difficulty—that of locating nest sites (Castilla and Swallow, '96). In addition, the combined effects of multiple environmental factors on incubation may mask the effects of temperature on offspring fitness. Incubation of eggs at constant temperatures in the laboratory does not provide ecologically relevant data about the thermal requirements and temperature limits for eggs in nature, but this approach provides an opportunity to test the subtle effects of any given temperature on eggs and hatchlings.

Calotes versicolor is a medium sized [to 105 mm snout-vent length (SVL)], semi-arboreal diurnal agamid lizard, and female individuals in Tongshi (18° 47′N, 109° 30′E), where our lizards were collected, lay multiple clutches with 3–14 eggs each depending on maternal SVL (Ji et al., in press). *C. versicolor* has a wide distributional range, which covers southeastern Iran to Afghanistan and Nepal, India to Sri Lanka, Burma to Indochina, southern China to Peninsular Malaysia and Sumatra (Zhao and Adler, '93). The lizard is thought to be extremely successful in some parts of its range as on Singapore Island, where the introduced *C. versicolor* has been displacing a native agamid lizard, *Bronchocela cristatella* (Diong et al., '94). Previous work on egg incubation in *C. versicolor* only showed data of incubation period and hatchling size (SVL and total length) of eggs incubated in an unknown thermal regime (Diong et al., '94). So, the extent to which incubation thermal environments affect eggs and hatchlings remains unclear. In this study, we incubated eggs at four constant temperatures ranging from 24°C to 33°C. We fixed the lower level of incubation temperature at 24°C and the upper level at 33°C, because the annual mean and maximal temperatures in Tongshi are approximately 23°C and 33°C, respectively. Our aims were to test whether incubation temperature affects the following four traits that we judged as likely to influence offspring fitness: (1) hatching success; (2) duration of incubation; (3) energy expenditure for embryonic development; and (4) size and several morphological traits of hatchlings.

MATERIALS AND METHODS

Eggs were obtained from 20 gravid females collected from March 1999 to May 1999 from a population of *C. versicolor* in Tongshi, Hainan, southern China. Females were maintained in $3 \times 1.5 \times 2.2$ m^3 (length × width × height) wire enclosures in our laboratory at Hangzhou Normal College (Hangzhou, Zhejiang, eastern China). Temperatures inside the terrarium were never below 23°C, and supplementary heating with suspended lamps

was provided such that lizards had ample opportunities for behavioral thermoregulation during the photophase. We fed lizards with mealworms (larvae of *Tenebrio molitor*), commercial worms, and water enriched with vitamins and minerals. Oviposition occurred between 20 April 1999 and 15 June 1999, and all freshly laid eggs were measured, weighed, and numbered promptly to minimize changes in egg mass at oviposition due to loss or gain of water.

Composition of eggs

One or two freshly laid eggs from each clutch were opened at laying. Each dissected egg contained a small embryo, which was too small and fragile to be separated from the yolk. Egg contents (yolk, including embryo) were removed and weighed. Shells were rinsed in distilled water, dried by blotting with a paper towel, and weighed. Egg contents and shells were dried in an oven to constant mass at 65°C, weighed, and stored frozen for later determination of their composition.

Nonpolar lipids in dried samples of egg contents were extracted in a Soxhlet apparatus for a minimum of 5.5 hr using absolute ether as solvent. The amount of lipids in each sample was calculated by subtracting the lipid-free dry mass from the total sample dry mass. We determined ash and energy in egg contents using a GR-3500 adiabatic bomb calorimeter (Changsha Instruments, China). We determined the inorganic material of eggshells by burning them in a muffle furnace at 700°C for a minimum of ten hours and weighing the remaining ash.

Incubation of eggs

We incubated the remaining eggs systematically (such that eggs from single clutches were distributed as equally as possible among treatments) at 24°C, 27°C, 30°C, and 33 (± 0.3) °C in plastic containers (250 × 180 × 70 mm) (length × width × height) that were covered with a perforated plastic membrane to retard water loss. All eggs were incubated at the same moisture level at all temperatures [2 g water/1 g vermiculite; producing approximately –12 kPa water potential (Lin and Ji, '98; Ji and Braña, '99)]. Eggs were half-buried in the substrate throughout the course of incubation, with the surface near the embryo exposed to air inside the container. We moved the containers among shelves in the incubator daily following a predetermined schedule to minimize any influence of thermal gradient inside the incubator. Incubation temperature was monitored twice daily using a digital thermometer. We weighed containers daily and, if necessary, added water to the vermiculite to compensate for small evaporative losses and water absorbed by eggs, thereby maintaining the substrate moisture content constant. Eggs were weighed at 5-day intervals. When eggs were found to have pipped, they were moved individually into petri dishes and covered, thereby ensuring positive identification of emergent young. The duration of incubation, measured as the number of days to pipping, was recorded for each egg. Upon emergence, each hatchling was weighed and then killed by freezing to –15°C for later study.

Size, morphology, and composition of hatchlings

A total of 73 frozen hatchlings were later thawed for data collection. Most ($N = 58$) of these hatchlings were alive at hatching, but 15 of 16 measured hatchlings incubated at 33°C died at the last stage of incubation solely because they were unable to pip out of the egg. All hatchlings that died at the last stage of incubation were well-developed, with residual yolks having been reabsorbed. Morphological measurements taken for each hatchling included: SVL, tail length, head length (from the snout to the anterior edge of tympanum), head width (taken at the posterior end of the mandible), fore- (humerus plus ulna) and hind- (femur plus tibia) limb lengths, tympanum diameter, and eye diameter. Sex of hatchlings was determined by pressing on both sides of the tail base using forceps for the presence or absence of hemipenes; the presence of hemipenes allowed unequivocal sex assignation of males (Ji and Braña, '99). After taking the measurements, each hatchling was separated into two components, carcass (including fat bodies), and residual yolk. Both components were dried in an oven at 65°C to constant mass, weighed, and preserved for later determination of composition, following the aforementioned procedures for eggs. Energy expenditure for embryonic development was determined indirectly by the difference in energy contents between egg contents of the freshly laid egg and the hatchling. Left-right symmetry was studied by evaluating four morphometric traits that were measured on both sides of each animal: fore- and hindlimb lengths, tympanum diameter, and eye diameter.

Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test, and for homogeneity of

variances using Bartlett's test. Log_e transformation was performed when necessary to satisfy the conditions for using parametric tests. We use linear regression analysis, one- and two- way analyses of variance (ANOVA), and one- and two-way analyses of covariance (ANCOVA), when the assumptions of parametric analyses were met. Nonparametric analyses were used when these assumptions were violated. The homogeneity of slopes was checked prior to testing for differences in the adjusted means. A principal component analysis (varimax rotation) was used to investigate the possible existence of morphological space characteristic of hatchlings from different incubation temperatures. As our focus was on variation in shape, we used size-corrected values (residuals from the regression on SVL) for all the variables. Differences in fluctuating asymmetry among incubation temperatures were tested using Levene's tests to compare the variances of the signed differences to the measurements from each side, scaled by individual $[(R_i - L_i)/(R_i + L_i)/2;$ Palmer and Strobeck, '86]. Throughout this paper, values are presented as mean ± one standard error, and the significance level is set at $\alpha = 0.05$.

RESULTS

C. versicolor lays pliable-shelled eggs. The clutch mean mass of eggs was 0.52 ± 0.01 g ($N = 20$, range = 0.45–0.61 g). Eggs incubated at different temperatures did not differ in mean initial mass ($F_{3,69} = 0.35$, $P = 0.787$). The mean initial mass of eggs incubated at 24°C, 27°C, 30°C, and 33°C was 514.7 mg, 513.3 mg, 501.1 mg, and 507.9 mg, respectively; the overall mean egg mass was approximately 510 mg.

Temporal changes in egg mass

In all temperature treatments, eggs steadily gained mass during incubation, and mass gains were a result of absorption of water (Fig. 1). Eggs incubated at higher temperatures gained mass (water) faster than eggs incubated at lower temperatures (Fig. 1). Except for eggs incubated at 27°C, the final (pre-hatching) egg mass was not affected by the initial egg mass (all $P > 0.06$). Therefore, the covariate was eliminated from consideration and an ANOVA (with temperature as the factor) was performed. Incubation temperature significantly affected the final egg mass ($F_{3,70} = 10.82$, $P < 0.0001$), with eggs incubated at 33°C finally gaining more mass than eggs incubated at 27°C and 30°C (Tukey's test, both $P < 0.0002$; Fig. 1). The final mass was apparently greater in eggs
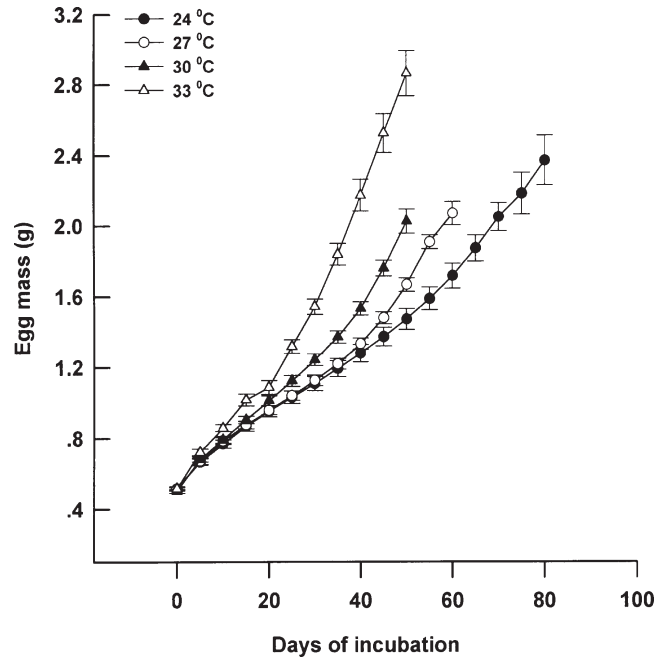

Fig. 1. Temporal changes in mass of *Calotes versicolor* eggs incubated at different temperatures. Data are expressed as mean ± one standard error.

incubated at 24°C than in those at 27°C and 30°C (Fig. 1), but the differences were not significant (Tukey's test, both $P > 0.05$).

Duration of incubation, hatching success, and sex ratio of hatchlings

As only one egg hatched at 33°C, the related datum on duration of incubation was eliminated from further statistical analysis. Duration of incubation varied considerably among temperature treatments ($F_{2,54} = 991.04$, $P < 0.0001$), and it decreased dramatically as temperature increased. For every 3°C increase in temperature from 24°C to 33°C, the incubation length decreased 21.6 days, 9.1 days, and 1.1 days, respectively (Table 1).

Incubation temperature significantly affected hatching success (*G*-test, $G = 28.41$, $df = 3$, $P < 0.001$). Hatching success was apparently greater at 27°C and 30°C and much lower at 24°C and 33°C (Table 1). Incubation temperature did not affect the sex ratio of hatchlings ($G = 6.84$, $df = 3$, $P > 0.10$), and the overall sex ratio (females/males = 33/40; Table 1) did not differ from equality ($G = 0.67$, $df = 1$, $P > 0.25$).

Of the 60 fertile eggs that failed during incubation, 27 embryos died at an early stage (within five days after laying), 22 died at a later stage (1–5 days prior to hatching, with yolk sac either absorbed or unabsorbed), and only one (at 33°C)

TABLE 1. *The effects of incubation temperatures on duration of incubation, hatching success, and sex ratio and abnormality of hatchlings in the oriental garden lizard,* Calotes versicolor[1]

Temperature (°C)	Incubated eggs	Incubation length (d)	Hatching success (%)	Sex ratio[2] (♀♀/♂♂)	Abnormality[2] (%)
24	30	82.1 ± 0.6 (78.9–85.7)	43.3 (13/30)	11/2	3.3 (1/30)
27	31	60.5 ± 0.4 (56.4–65.9)	80.6 (25/31)	10/15	12.9 (4/31)
30	28	51.4 ± 0.5 (46.3–55.2)	67.9 (19/28)	4/15	10.7 (3/28)
33	29	50.3[3]	3.4 (1/29)	8/8	58.6 (17/29)

[1]Data on duration of incubation are expressed as mean ± one standard error (range).
[2]Including the individuals that died in the last stage of incubation.
[3]Only one egg hatched at 33°C.

died midway during incubation. Embryos that died at the last stage of incubation were all well-developed, with residual yolks having been internalized. A total of 25 hatchlings exhibited trunk and/or tail malformations. Deformed individuals were found in each temperature treatment, with significantly more deformities observed at 33°C ($G = 22.87$, $df = 3$, $P < 0.001$; Table 1).

Size, mass, and composition of hatchlings

Preliminary two-way ANOVAs (with sex and temperature as the factors) did not reveal differences between sexes in size (SVL and tail length), body mass, and composition of hatchlings (all $P > 0.05$), so we pooled data for both sexes (Table 2). Incubation temperature significantly affected wet body mass ($F_{3,69} = 2.82$, $P = 0.045$), dry body mass ($F_{3,69} = 3.45$, $P = 0.021$), carcass dry mass ($F_{3,69} = 10.52$, $P < 0.0001$), and residual yolk dry mass ($F_{3,69} = 3.33$, $P = 0.025$) of hatchlings. Hatchlings incubated at 24°C, 27°C, and 30°C did not differ in size, mass, and components, whereas hatchlings incubated at 33°C had greater wet body mass and residual yolk dry mass but smaller dry body mass and carcass dry mass than did those incubated at lower temperatures (Table 2). Incubation temperature also affected SVL ($F_{3,69} = 3.19$, $P = 0.029$) and tail length ($F_{3,69} = 3.12$, $P = 0.032$) of hatch-

lings, with hatchlings incubated at 27°C being significantly larger in both SVL and tail length than those incubated at 33°C (Table 2).

To evaluate the effects of incubation temperature on expenditure of energy and nutrients for embryonic development, we adjusted means for composition of hatchlings by ANCOVA, using initial egg mass as the covariate. The effects of incubation temperature on composition of hatchlings were very pronounced, as adjusted means for total dry mass, energy, lipids, and ash varied considerably among temperature treatments (Table 3). It is worth noting that hatchlings incubated at 33°C were smaller in all adjusted means examined whereas those incubated at three lower temperatures did not differ in any of these adjusted means (Table 3).

Expenditure of energy and nutrients for embryonic development

Freshly laid eggs and hatched eggs did not differ in shell dry mass (one-way ANCOVA, $F_{4,84} = 1.17$, $P = 0.329$), and dried shells accounted for approximately 4.0% of the total egg wet mass at hatching. Dried shells from freshly laid eggs had a higher percentage of ash ($21.0 \pm 0.4\%$, $N = 16$, range = 18.3–22.4%) than did those from hatched eggs (10.2–12.6%, depending on temperature

TABLE 2. *The effects of incubation temperature on body mass, snout-vent length, and tail length of* Calotes versicolor *hatchlings*[1]

Temperature (°C)	N	Body mass (mg)	Hatchling dry mass (mg)			Snout-vent length (mm)	Tail length (mm)
			Dry body mass	Carcass	Residual yolk		
24	13	517.9[ab] ± 16.4 (450.4–675.0)	103.7[ab] ± 4.0 (82.6–139.1)	93.0[a] ± 3.8 (71.5–120.9)	10.7[ab] ± 1.2 (5.3–20.8)	24.7[ab] ± 0.3 (23.2~26.6)	46.6[ab] ± 1.9 (38.3~59.5)
27	25	553.3[b] ± 14.0 (398.1–723.5)	107.5[a] ± 2.9 (86.0–148.4)	98.7[a] ± 2.9 (73.1–140.1)	8.8[b] ± 0.9 (1.0~22.5)	24.6[a] ± 0.2 (22.5~27.1)	47.0[a] ± 1.3 (36.0~59.5)
30	19	555.3[ab] ± 17.3 (425.3–714.0)	108.3[a] ± 3.9 (82.1–148.4)	96.5[a] ± 3.9 (63.2–137.5)	11.8[ab] ± 1.6 (3.4~36.0)	24.3[ab] ± 0.2 (22.4~26.2)	46.5[ab] ± 1.3 (38.5~56.0)
33	16	630.4[a] ± 29.8 (421.9–841.6)	91.9[b] ± 5.5 (58.5–144.5)	73.0[b] ± 3.8 (41.1~110.2)	21.2[a] ± 0.5 (3.9~66.3)	23.4[b] ± 0.5 (19.4~25.8)	41.6[b] ± 1.4 (32.8~50.0)

[1]Data are expressed as mean ± one standard error (range). Means with different superscripts differ significantly (Tukey's test, α = 0.05; a>b).

TABLE 3. *The effects of incubation thermal and hydric environments on energy contents, lipid mass, and ash mass of* Calotes versicolor *hatchlings and dry mass and ash mass of shells from hatched eggs*[1]

Temperature (°C)	N	Dry mass (mg)	Energy (KJ)	Nonpolar lipids (mg)	Ash mass (mg)
24	13	102.6 ± 2.5	2.27 ± 0.09	15.1 ± 0.5	9.5 ± 0.2
27	25	106.7 ± 2.0	2.37 ± 0.06	17.1 ± 0.3	9.8 ± 0.2
30	19	110.3 ± 3.2	2.40 ± 0.09	18.0 ± 0.8	10.0 ± 0.3
33	16	92.4 ± 4.9	2.07 ± 0.12	13.1 ± 1.1	8.3 ± 0.3
$F_{3,68}$		5.66**	4.79**	9.08***	8.43***
		24[ab], 27[a], 30[a], 33[b]	24[ab], 27[a], 30[a], 33[b]	24[ab], 27[a], 30[a], 33[b]	24[a], 27[a], 30[a], 33[b]

[1]Data are expressed as adjusted mean ± one standard error. F values of ANCOVAs (with initial egg mass as the covariate) are indicated in the table. Symbols immediately after *F* values represent significant levels: **$P < 0.01$, ***$P < 0.001$. Adjusted means with different superscripts differ signficantly (Tukey's test, α = 0.05; a>b).

treatments). This difference suggests that inorganic material may be moved from the eggshell to developing embryo for skeletogenesis, and it provides evidence that embryos of *C. versicolor* use eggshells as the secondary source of inorganic material for development.

When egg mass is kept constant at 510 mg, we found that the quantities of dry material, energy, lipids, and ash in the egg contents of freshly laid eggs *(N = 16)* were 182.0 ± 3.8 mg, 4.72 ± 0.10 KJ, 61.7 ± 1.4 mg, and 9.8 ± 0.2 mg, respectively. During incubation, 50.8–60.6% of dry material, 43.9–50.8% of energy, and 21.2–29.2% of nonpolar lipids in the egg contents of freshly laid eggs were transferred to the hatchling. The effects of incubation temperature on these conversion efficiencies were very pronounced, with eggs incubated at 33°C exhibiting the lowest conversion efficiencies (Table 3).

Morphology and bilateral asymmetry of hatchlings

As preliminary two-way ANCOVAs (with sex and incubation temperature as the factors and SVL as the covariate) revealed between-sex differences in several morphological traits of hatchlings, we examined the effects of sex and incubation temperature and their interactions on variation in morphological traits of hatchlings (Table 4). When removing the effects of differences in SVL, we found that the body mass and head width were greater in male hatchlings, but the remaining hatchling traits did not differ between sexes (ANCOVAs; Table 4). Incubation temperature sig-

TABLE 4. *Morphological phenotypes of* Calotes versicolor *hatchlings, according to sex and incubation temperature*[1]

Hatchling traits		Incubation temperature (°C)				Effects		
		24	27	30	33	Sex $F_{1, 64}$	Temperature $F_{3, 64}$	Interaction $F_{3, 64}$
SVL (mm)	M	24.4 ± 0.3	24.6 ± 0.2	24.2 ± 0.3	22.6 ± 0.7	2.29 ns	3.13*	1.08 ns
	F	24.7 ± 0.3	24.7 ± 0.4	24.7 ± 0.2	24.2 ± 0.5		24[ab], 27[a], 30[ab], 33[b]	
Body mass	M	583.6 ± 36.6	551.2 ± 17.9	560.4 ± 21.5	663.0 ± 43.6	5.14*	9.22***	2.29 ns
(mg)	F	569.8 ± 18.8	556.6 ± 23.8	536.0 ± 18.1	597.7 ± 40.0	M>F	24[b], 27[b], 30[b], 33[a]	
Tail length	M	43.9 ± 0.4	49.1 ± 1.4	47.3 ± 1.4	39.3 ± 1.9	280 ns	0.32 ns	2.26 ns
(mm)	F	47.1 ± 2.2	43.6 ± 1.9	43.2 ± 2.1	43.8 ± 2.0			
Head length	M	7.0 ± 0.02	7.3 ± 0.1	7.1 ± 0.1	6.7 ± 0.2	3.21 ns	5.34**	2.38 ns
(mm)	F	7.2 ± 0.1	7.2 ± 0.1	7.0 ± 0.1	6.8 ± 0.03		24[a], 27[a], 30[a], 33[b]	
Head width	M	5.7 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	12.21***	1.28 ns	2.32 ns.
(mm)	F	5.6 ± 0.1	5.6 ± 0.1	5.4 ± 0.02	5.4 ± 0.1	M>F		
Forelimb length	M	8.1 ± 0.01	8.2 ± 0.1	8.2 ± 0.1	7.6 ± 0.1	3.71 ns	1.61 ns	1.87 ns
(mm)	F	8.2 ± 0.1	8.2 ± 0.2	8.1 ± 0.1	7.7 ± 0.1			
Hindlimb length	M	11.7 ± 0.5	12.1 ± 0.2	11.7 ± 0.2	10.3 ± 0.4	0.41 ns	13.15***	0.79 ns
(mm)	F	12.2 ± 0.2	12.0 ± 0.3	11.5 ± 0.1	10.8 ± 0.2		23[a], 27[a], 30[a], 33[b]	
Tympanum	M	1.1 ± 0.04	1.2 ± 0.02	1.1 ± 0.01	1.0 ± 0.04	0.51 ns	10.63**	0.25 ns
length (mm)	F	1.2 ± 0.02	1.2 ± 0.02	1.1 ± 0.03	1.1 ± 0.02		24[a], 27[a], 30[a], 33[b]	
Eye length	M	1.8 ± 0.04	1.9 ± 0.02	1.8 ± 0.02	1.8 ± 0.04	0.08 ns	4.06**	0.23 ns
(mm)	F	1.9 ± 0.02	1.9 ± 0.04	1.8 ± 0.02	1.7 ± 0.03		24[a], 27[a], 30[ab], 33[b]	

[1]F values correspond to single effects and factor interactions in two-factor ANOVA (for snout-vent length, SVL) or ANCOVAs (with SVL as the covariate, for other hatchling traits). Data are expressed as mean ± one standard error. Symbols immediately after *F* values represent signficant levels: ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Means with different superscripts differ significantly (Tukey's test, α = 0.05; a>b).

nificantly affected such hatchling phenotypes as body mass, head length, hindlimb length, tympanum diameter, and eye diameter, but it did not affect tail length, head width, and forelimb length (ANCOVAs; Table 4). For all morphological traits showing significant differences among temperature treatments, hatchlings incubated at 33°C were smaller (SVL), heavier (body mass relative to SVL), and shorter (head length, hindlimb length, tympanum diameter, and eye diameter relative to SVL) than those incubated at lower incubation temperatures (Table 4). Hatchlings incubated at the three lower incubation temperatures did not differ in any of the examined morphological traits (Table 4).

A principal component analysis resolved two components (with eigenvalues > 1) from eight size (SVL)-free morphological variables, accounting for 60.6% of variation in the original data (Table 5). The first component (43.0% variance explained) had high positive loading for size-free values of head dimension (length and width) and fore- and hindlimb lengths, and the second component (17.6% variance explained) had high positive loading for size-free value of body mass. Hatchlings from different incubation temperatures differed in their scores on the first ($F_{3,68}$ = 4.96, P < 0.004; 24^a, 27^a, 30^{ab}, 33^b, Tukey's test, a > b) and second axes ($F_{3,68}$ = 10.52, P < 0.0001; 24^b, 27^b, 30^{ab}, 33^b, Tukey's test, a > b) (Fig. 2).

The scaled differences between measurements of forelimb length ($F_{3,69}$ = 0.12, P = 0.946), hindlimb length ($F_{3,69}$ = 0.27, P = 0.844), and tympanum diameter ($F_{3,69}$ = 0.12, P = 0.946) of both sides did not exhibit heterogeneity of variances among temperature treatments. Heterogeneity of variances among temperature treatments existed only for the scaled difference between measurements of eye di-
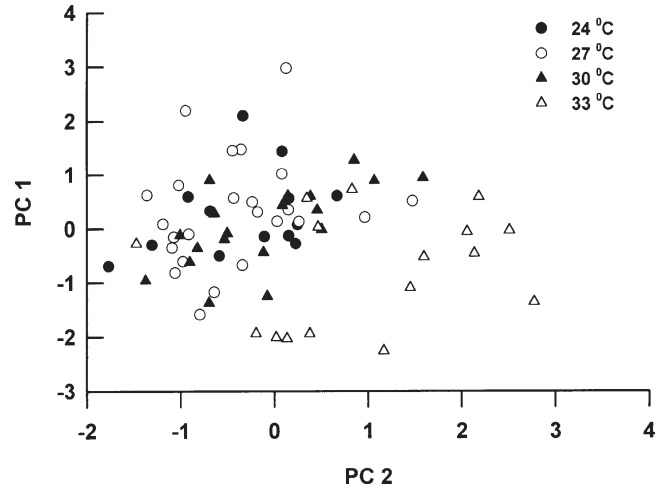

Fig. 2. Positions of *Calotes versicolor* hatchlings from different incubation temperatures (symbols on the top right corner) in the space defined by the first two axes of a principal component analysis based on eight, size-adjusted morphological variables, on which size effects were removed by using residuals from the regressions on snout-vent length.

ameter of both sides ($F_{3,69}$ = 3.06, P = 0.034). The magnitude of the variances, and hence the level of asymmetry, of eye diameter increased as temperature increased. This suggests that the highest incubation temperature induces the highest level of asymmetry in eye diameter in *C. versicolor*.

DISCUSSION

Eggs of *C. versicolor* in all laboratory temperature treatments gained mass throughout incubation, but eggs incubated at 24°C and 33°C gained apparently more mass than those incubated at 27°C and 30°C (Fig. 1). These observations indicate that water exchanges between eggs and their surroundings are dependent on the thermal conditions encountered by incubating eggs, and the hydric environment inside the egg can be very different when eggs are incubated in different thermal environments. However, there is no direct evidence to support the hypothesis that the hydric conditions experienced by embryos of *C. versicolor* during embryogenesis significantly affect their developmental conditions at hatching. Hatchlings (N = 16) incubated at 33°C were heavier in wet body mass than those incubated at lower temperatures, largely because this group of hatchlings included 15 well-developed individuals that died at the last stage of incubation. As dead individuals are unable to osmoregulate, it is not surprising that full-term embryos that failed to hatch (dead-in-shell hatchlings) contain relatively more water, and hence are heavier in

TABLE 5. *Loading of the first two axes of a principal component analysis on eight morphological variables in* Calotes versicolor *hatchlings*[1]

Variable	Factor loading	
	PC1	PC2
Body mass	0.066	**0.897**
Tail length	0.513	0.100
Head length	**0.870**	0.002
Head width	**0.739**	0.221
Foreleg length	**0.813**	0.022
Hindleg length	**0.851**	−0.341
Tympanum length	0.569	−0.602
Eye length	0.397	−0.261
Variance explained	43.0%	17.6%

[1]Size effects are removed in all cases by using residuals from the regressions on snout-vent length. Variables with the main contribution to each factor are in bold face.

wet mass than viable hatchlings. Actually, hatchlings incubated at 24°C, 27°C, and 30°C did not differ significantly in any of the examined traits, although the hydric conditions inside the egg were different among eggs incubated at these temperatures (Fig. 1). We conclude from these results that embryonic development in *C. versicolor* is relatively insensitive to variation in hydric environments during incubation. This conclusion is supported by studies on other squamates that also lay pliable-shelled eggs (e.g., Gutzke and Packard, '87; Lin and Ji, '98; Ji and Braña, '99; Braña and Ji, 2000; Ji and Du, 2001a,b; Ji and Zhang, 2001; Ji et al., 2001).

All incubation thermal conditions used in this study yielded an equal sex ratio (Table 1). There was no evidence of gender difference in phenotypic responses to incubation temperature (i.e., significant interactions between sex and incubation temperature for phenotypes examined) (Table 4). However, incubation temperature profoundly affects many aspects of egg incubation in *C. versicolor*, including the aforementioned temporal changes in egg mass, hatching success, expenditure of energy and nutrients for embryonic development, and several hatchling traits examined in this study. Therefore, our data add evidence to previous studies demonstrating pervasive effects of incubation temperature on developmental rates and offspring phenotypes in ectotherms, including reptiles (Deeming and Ferguson, '91; Van Damme et al., '92; Shine and Harlow, '93; Atkinson, '94; Overall, '94; Johnston et al., '96; Ji and Braña, '99; Braña and Ji, 2000; Ji and Du, 2001a,b; Ji and Zhang, 2001; Ji et al., 2001).

Significant differences in body linear dimension were found among hatchlings of *C. versicolor* from eggs incubated at different temperatures, with hatchlings incubated at 33°C having shorter SVL and tail length than those incubated at lower temperatures (Tables 2 and 4). Our finding that eggs incubated at moderate temperatures produce larger hatchlings is consistent with that reported for other reptiles (Gutzke et al., '87; Packard et al., '89; Burger, '90; Van Damme et al., '92; Lin and Ji, '98; Ji and Braña, '99; Braña and Ji, 2000; Ji and Du, 2001a,b; Ji and Zhang, 2001; Ji et al., 2001). As the size of hatchlings is closely associated with the size of carcasses (Ji et al., '97, '99a,b, 2001; Ji and Sun, 2000; Ji and Du, 2001a,b), the smaller size of hatchlings (full-term embyros) incubated at 33°C is therefore primarily attributed to their smaller carcasses (Table 2). However, size at hatching may not mirror the ultimate size to

which newly emerged hatchlings will grow prior to feeding, because hatchlings use the resources in the residual yolk to increase their size during the first post-hatching days (e.g., Packard, '91; Ji et al., '97, '99a,b; Ji and Braña, '99; Ji and Sun, 2000). Previous studies indicate that eggs of reptiles incubated at high temperatures leave more unutilized yolk at hatching and, consequently, produce smaller hatchlings (Beuchat, '88; Phillips et al., '90; Phillips and Packard, '94; Ji and Braña, '99; Ji and Du, 2001a,b; Ji and Zhang, 2001; Ji et al., 2001). *C. versicolor* also shares this feature, as hatchlings incubated at 33°C had smaller carcasses but larger residual yolks than did hatchlings derived from eggs incubated at lower temperatures (Table 2). Residual yolks in hatchlings of *C. versicolor* are far from trivial (Table 2), so we expect that pronounced post-hatching growth of hatchling following the reabsorption and utilization of residual yolk would occur in this species. However, it is very unlikely for any of the hatchlings incubated at 33°C to grow to a size that approximates the size of hatchlings incubated at lower temperatures merely through the mobilization of the resources in the residual yolk, because of the higher energy expenditure for embryogenesis at 33°C (Tables 2,3).

Hatchling size has been thought to be an important determinant of fitness in many lizards, and larger size may have several advantages (Van Damme et al., '92; Galán, '96; Braña and Ji, 2000). Larger young are more successful at hunting prey and avoiding predators (Christian and Tracy, '81; Avery et al., '82; Webb, '86; Sinervo and Adolph, '89; Ji and Zhang, 2001). Moreover, larger young may have prior access to limited resources and better chances to survive the first active season and winter (Fox, '78; Ferguson et al., '82; Ferguson and Fox, '84; Garland et al., '90). Accordingly, the smaller sized hatchlings of *C. versicolor* obtained from eggs incubated at 33°C suggest that these hatchlings have a lower fitness compared to hatchlings derived from eggs incubated at lower temperatures.

The remaining morphological traits examined in this study, relative (to SVL) head length, hindlimb length, tympanum diameter, and eye diameter, showed significant treatment effects. Relative head width was the only trait that was sexually dimorphic (Table 3). Hatchlings from eggs incubated at 33°C had relatively shorter head length relative to SVL (Table 3), and hence smaller heads than those incubated at the three lower temperatures. A larger head is thought to be as-

sociated with an increased ability of a predator to eat larger prey (e.g., Schoener et al., '82; Shine, '91; Barden and Shine, '94). Moreover, according to optimal foraging models, a predator tends to increase the rate of net energy intake by consuming larger prey items so as to maximize net energy gain. Thus, any relative decrease in head size induced by incubation temperature decreases hatchlings' potential to feed larger prey, survivorship and growth, and thereby reduces overall fitness in the offspring.

Hindlimb length is thought to be associated with locomotor performance in lizards (Miles, '94; Bauwens et al., '95; Miles et al., '95; Braña and Ji, 2000), so this trait could be ecologically important. Unfortunately, hatchlings of *C. versicolor* did not run as well as hatchlings of ground-dwelling lizards [e.g., *Podarcis muralis* (Van Damme et al., '92; Braña and Ji, 2000), *Takydromus septentrionalis* (Lin and Ji, '98), and *Eumeces chinensis* (Ji and Zhang, 2001)], because they always tried to climb the walls of the racetrack when chased. So, the extent to which the reduced hindlimb length in hatchlings incubated at extremely high temperatures affects their locomotor performance remains unclear.

Eye diameter relative to SVL was shorter in hatchlings incubated at 33°C, but it did not differ among hatchlings incubated at different temperatures when the influence of head size difference was removed (ANCOVA with head length as the covariate: $F_{3,68} = 2.21$, $P = 0.094$). Therefore, the shorter eye diameter of hatchlings incubated at 33°C is primarily attributed to smaller head size. On the contrary, tympanum diameter was shorter in hatchlings incubated at 33°C even when the effects of head size differences were removed (ANCOVA with head length as the covariate: $F_{3,68} = 6.83$, $P < 0.001$). Eye and tympanum diameters are both bilateral traits presumably associated with sensor functions of lizards. However, influence of temperature-induced modifications to the two traits on fitness remains unclear at this time.

Bilateral symmetry mirrors to a large extent developmental stability and is positively correlated with several fitness components (Møller, '97; Møller and Swaddle, '97; but see Clarke, '98). Asymmetry is thought to be associated with environmental stress during development, so that a high degree of asymmetry should mirror unsuitable developmental conditions and identify comparatively unbalanced phenotypes (Palmer and Strobeck, '86; Leary and Allendorf, '89; Braña and Ji, 2000). High, bilateral asymmetry could impair

the normal functions of bilateral organs and hence is an overall indicator of poor quality phenotypes. Therefore, the existence and level of fluctuating asymmetry among hatchlings from different incubation temperatures could be used as an indicator of hatchling quality. In this study, of the four bilateral traits examined, only eye diameter showed an asymmetry that was temperature dependent—the level of asymmetry was highest at the highest incubation temperature. Thus, our results provide evidence of temperature-mediated asymmetry of bilateral traits during embryogenesis, with the highest incubation temperature producing the highest level of asymmetry.

Taken together, our results show that incubation temperature significantly affects, but not in a continuous way, most aspects of egg incubation in *C. versicolor*. This study further demonstrates the importance of incubation temperature as a source of phenotypic variation in lizards. Overall, eggs of *C. versicolor* can be incubated at constant temperatures within the range from 24°C to 30°C without significant modifications to hatchling phenotypes, suggesting that influence of temperature is well-buffered with this range. Exposure of eggs to lower incubation temperatures slows or arrests embryonic development but has little or no observable lethal effect on embryos (Andrews and Rose, '94; Sexton and Marion, '74; Shine and Harlow, '96; Andrews et al., '97). The lower hatching success at 24°C can be attributed to the prolonged exposure (82 days) of eggs to the effects of adverse biotic (increased microbial contamination) and abiotic (fluctuating wetness and temperatures) factors in the incubation environment of the eggs, which may substantially reduce hatching success. On the contrary, extremely high temperatures have a lethal effect on developing embryos. In this study, the upper critical threshold seems to be close to 33°C, because hatchlings incubated at 33°C showed differences in composition and morphological traits versus those incubated at lower temperatures. The existence of the upper threshold of constant incubation temperature for the main detrimental effects is consistent with results reported in previous studies on squamate reptiles (Van Damme et al., '92; Lin and Ji, '98; Ji and Braña, '99; Braña and Ji, 2000; Ji and Du, 2001a,b; Ji and Zhang, 2001; Ji et al., 2001). Phenotypic variation induced by incubation temperatures could be potentially important for lizards living in natural environments, but the extent to which environment-mediated phenotypic variation affects offspring fitness in nature remains largely

unknown, and it offers considerable potential for future work.

ACKNOWLEDGMENTS

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