

Incubation temperature affects hatchling growth but not sexual phenotype in the Chinese soft-shelled turtle, *Pelodiscus sinensis* (Trionychidae)

Xiang Ji^{1,2*}, Fang Chen², Wei-Guo Du² and Hui-Li Chen²

¹ School of Life Sciences, Nanjing Normal University, Nanjing 210097, Jiangsu, People's Republic of China

² School of Life Sciences, Hangzhou Normal College, Hangzhou 310036, Zhejiang, People's Republic of China

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Abstract

Eggs of *Pelodiscus sinensis* were incubated under one fluctuating and four constant temperatures, and hatchlings from different incubation temperatures were maintained under identical conditions to assess the effects of incubation temperature on sexual phenotype and hatchling growth. The incubation length decreased as temperature increased, but it did not differ between sexes within each temperature treatment. Hatching success was higher at intermediate temperatures (28 °C, 30 °C and the fluctuating temperature regime) than at low (24 °C) and high (34 °C) temperatures. The sex ratio of hatchlings did not differ from equality within each temperature treatment. Thus, our data support previous work that *P. sinensis* does not have temperature-dependent sex determination, and add evidence for the prediction that turtles within the Trionychidae have genotypic sex determination exclusively. Incubation temperature affected hatchling mass, with hatchlings from intermediate incubation temperatures being heavier than those from low (24 °C) and high (34 °C) incubation temperatures. Hatching size was not a predictor of post-hatching growth. Incubation temperature affected hatchling growth, with hatchlings from 24 °C overall growing faster than did hatchlings from higher incubation temperatures. The influence of incubation temperature on hatchling growth was well buffered within the range of constant temperatures from 28 °C to 34 °C. Fluctuating incubation temperatures augmented male growth but reduced female growth, as female embryos were more vulnerable to extremely high temperatures.

Key words: turtle, *Pelodiscus sinensis*, egg incubation, sexual phenotype, growth

INTRODUCTION

Temperature regimes experienced by reptilian embryos affect not only hatching success, rates of development and embryonic use of energy, but also some phenotypic attributes of the hatchling including its morphology (Gutzke & Packard, 1987; Overall, 1994; Allsteadt & Lang, 1995; Packard & Phillips, 1995; Booth, 1998, 2000; Lin & Ji, 1998; Ji, Xu & Lin, 1999; Braña & Ji, 2000; Pan & Ji, 2001; Ji, Huang *et al.*, 2002; Ji, Qiu & Diong, 2002), energy reserves (Ji & Braña, 1999; Rhen & Lang, 1999a; Ji & Du, 2001a,b), behaviour (Lang, 1987; G. J. W. Webb & Cooper-Preston, 1989; Burger, 1991, 1998), locomotor performance (Van Damme *et al.*, 1992; Braña & Ji, 2000; Ji & Zhang, 2001; Zhang & Ji, 2002), growth (Joanen, McNease & Ferguson, 1987; G. J. W. Webb & Cooper-Preston, 1989; Brooks *et al.*, 1991; Bobyn & Brooks, 1994; Rhen & Lang, 1995, 1999b; Booth, 1999; Braña &

Ji, 2000) and sex in species with temperature-dependent sex determination (TSD) (Deeming & Ferguson, 1991; Janzen & Paukstis, 1991; Ewert, Jackson & Nelson, 1994; Lang & Andrews, 1994; Viets *et al.*, 1994). There is now evidence of effects of incubation temperature on hatching success, incubation length, embryonic use of energy, hatchling size and early growth in the Chinese soft-shelled turtle, *Pelodiscus sinensis* (Choo & Chou, 1985, 1987; Du & Ji, 2001, 2003; Nie *et al.*, 2001), a species widely distributed in central and southern China and south-eastern Asia (Zhao & Adler, 1993). However, the effects of incubation temperature on hatchling growth and sexual phenotype, if any, remain unclear.

The influence of incubation temperature on an offspring's growth, particularly long-term growth, is a subject of general interest but only a few studies have addressed this topic (e.g. Joanen *et al.*, 1987; Brooks *et al.*, 1991; Van Damme *et al.*, 1992; McKnight & Gutzke, 1993; Bobyn & Brooks, 1994; Rhen & Lang, 1995, 1999b; O'Steen, 1998; Booth, 1999; Braña & Ji, 2000). In China, *P. sinensis* is an economically important turtle, which is widely cultured by local people for food. Hatchery

*All correspondence to: Xiang Ji, School of Life Sciences, Hangzhou Normal College, Hangzhou 310036, Zhejiang, People's Republic of China. E-mail: xji@mail.hz.zj.cn

operators usually incubate *P. sinensis* eggs at temperatures around 30°C, presumably because of relatively high hatching success and short incubation length at these temperatures. Whether these incubation temperatures are optimal for hatchling growth remains unknown. In addition, the mechanism of sex determination for *P. sinensis* still remains obscure: some researchers considered it to be genotypic sex determination (GSD) (Choo & Chou, 1985), while others considered it to be TSD (Zhu & Sun, 2000; Nie *et al.*, 2001).

In this study, *P. sinensis* eggs were incubated under five temperature regimes, with aims to answer the questions: does incubation temperature affect the sexual phenotype?; does size at hatching affect hatchling growth?; does hatchling growth differ between sexes?; does incubation temperature affect hatchling growth?

MATERIALS AND METHODS

Eggs ($n = 180$) from unknown numbers of mothers were collected within 12 h of oviposition from a private hatchery near Hangzhou (Zhejiang, eastern China), on 30 June 2001. These eggs were produced by *c.* 13 females, because the average clutch size of *P. sinensis* was 14.0 (7–27, $n = 46$). Eggs were brought to our laboratory in Hangzhou, where they were randomized to minimize the family effect, numbered individually with a pencil, measured (diameter) to nearest 0.1 mm with a digital calliper, and weighed to nearest 1 mg on a Mettler balance. Viable eggs could be easily identified by the presence of a white patch (indicative of fertilization) on the shell surface; infertile eggs ($n = 22$) were used to analyse egg composition and the data will be reported elsewhere. Most eggs ($n = 134$) were incubated at constant temperatures of 24, 28, 30 and 34 ± 0.3 °C in $25 \times 20 \times 10$ cm (length \times width \times height) vermiculite-filled plastic containers within LRH-250G incubators (Guangdong Medical Instrument, China), with the remaining eggs ($n = 24$) incubated under a fluctuating temperature regime in a vermiculite-filled plastic container of the same size within a $60 \times 60 \times 30$ cm chamber buried 40 cm below the ground surface in the exposed backyard of our laboratory. A Tinytalk datalogger (Gemini Pty, Australia) programmed to record temperature every 1 h was placed in the chamber throughout the experiment, so that temporal changes in temperature inside the chamber could be automatically recorded. Temperatures within the chamber varied daily and seasonally (Fig. 1), with the maximum magnitude of diel thermal variation, the mean temperature, the lowest temperature and the highest temperature being 8.0, 29.5, 23.7 and 36.2°C, respectively.

The containers holding the eggs were covered with a perforated plastic membrane to retard water loss. Eggs were half-buried in the substrate, with the white patch being kept upward. The water potential of the incubation substrate was held constant at -12 kPa water potential (2 g water/1 g vermiculite; Ji & Braña, 1999) for all

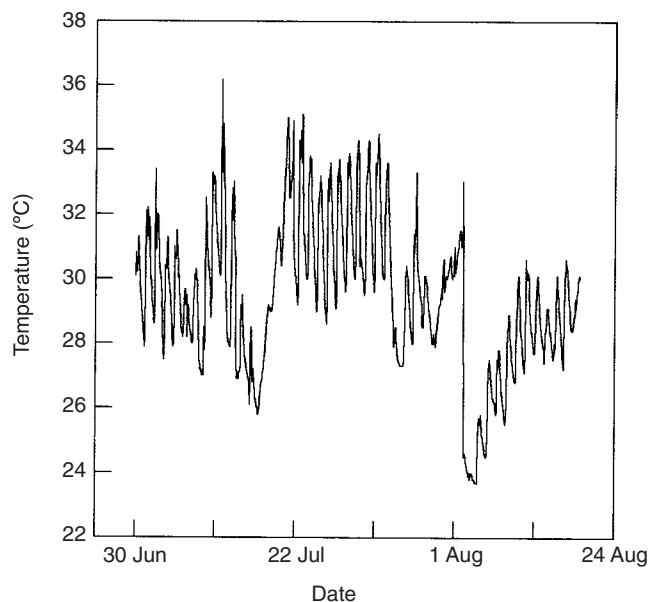


Fig. 1. Temporal variation in temperatures experienced by the *Pelodiscus sinensis* eggs incubated under the fluctuating temperature regime.

temperature treatments by weighing containers at intervals of 2 days and adding water to the vermiculite when necessary to compensate for small evaporative losses.

Upon emergence, hatchlings were weighed to the nearest 1 mg and marked individually by toe-clipping¹ for future identification. To control for any influence of temperature choice of turtles on hatchling growth that might have resulted from the experimental modification of the incubation temperature (O'Steen, 1998; but also see Brooks *et al.*, 1991; Bobyne & Brooks, 1994; Rhen & Lang, 1999b), hatchlings were maintained under identical thermal conditions. Turtles < 100 g ($n = 15$ –20) were housed in individual cages ($130 \times 90 \times 60$ cm) with 10 cm depth water, whereas turtles > 100 g ($n = 8$ –10) were housed in cages of the same size with 45 cm depth water. These cages were kept in a controlled temperature room at 30 ± 1 °C. The room lights were programmed to create a cycle of 12 h light:12 h dark. Turtles were fed commercial food (*c.* 10% water, 60% proteins, 5% lipids, 5% carbohydrates and 20% minerals) daily. Food was provided in an excess amount and spread throughout the cages, such that turtles had free access to the food. Turtles were weighed synchronously on 28 October 2001, 18 November 2001, 1 January 2002, 2 February 2002, 23 March 2002 and 23 May 2002, and those from incubation temperatures higher than 24°C were additionally weighed at 30 or 45 days of age. Turtles were sexed at 3 months of age, and re-sexed on 23 May 2002. Except for 2 individuals that

¹ Editor's note: The Ethical Committee of the Zoological Society of London considers that toe-clipping is no longer acceptable as a routine procedure for marking animals.

were later founded to be sexed wrongly, our diagnoses of gender in 3-month-old turtles were consistent with the results of sex determination at the end of the experiment.

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. Linear regression analysis, 1- and 2-way analysis of variance (ANOVA), and 1- and 2-way analysis of covariance (ANCOVA) were used when the assumptions of parametric analyses were met. Non-parametric analyses (G -test) were used when these assumptions were violated. The homogeneity of slopes was checked before testing for differences in the adjusted means. Tukey's test was used for multiple comparisons. Throughout this paper, values are presented as mean \pm 1 SE, and the significance level is set at $\alpha = 0.05$.

RESULTS

A two-way ANOVA (with sex and temperature as the factors) revealed that incubation length (\log_e transformed data) varied considerably among temperature treatments ($F_{4,106} = 1527.55$, $P < 0.0001$) but did not differ between sexes ($F_{1,106} = 3.66$, $P = 0.058$). The incubation length decreased dramatically as temperature increased (Table 1). Hatching success did not differ significantly among temperature treatments ($G = 2.43$, d.f. = 4, $P > 0.05$), but it was noticeably higher at the intermediate temperatures (28 °C, 30 °C and the fluctuating temperature regime) than at low (24 °C) and high (34 °C) temperatures (Table 1). The sex ratio of hatchlings did not differ from equality within each temperature treatment (G -test, $P > 0.10$ in all cases), although relatively more females were produced at 24 °C (67%) and 28 °C (54%) and fewer females were produced at 30 °C (36%) and 34 °C (41%) (Table 1). The overall sex ratio (females/males = 57/59; Table 1) did not differ from equality ($G = 0.03$, d.f. = 1, $P > 0.90$).

Eggs incubated in different thermal regimes did not differ in mean initial mass ($F_{4,112} = 2.01$, $P = 0.098$), and hatchling mass at hatching was positively correlated with initial egg mass in all treatments ($P < 0.01$ in all cases). A preliminary two-way ANCOVA (with sex and temperature as the factors and initial egg mass as the covariate) did not reveal differences between sexes in body mass at hatching ($F_{1,106} = 0.03$, $P = 0.859$), so data were pooled for both sexes within each temperature treatment. Incubation temperature affected body mass at hatching (ANCOVA: $F_{4,111} = 10.08$, $P < 0.0001$), with hatchlings incubated at 28 °C, 30 °C and fluctuating temperatures being heavier in body mass than those incubated at 24 °C and 34 °C (Table 2).

Body masses weighed at any of the six post-hatching occasions were independent of the mass at hatching within each temperature treatment, ($P > 0.07$ in all cases), so two-way ANOVA (with sex and temperature as the factors) was used rather than two-way ANCOVA to test for differences in body mass between sexes and

Table 1. Influence of incubation temperature on hatching success, duration of incubation and sex ratio of hatchlings for *Pelodiscus sinensis*. The thermal characteristics of the fluctuating temperature regime are expressed as the mean temperature and range. Sample sizes for sex ratio are less than those for hatching because of mortality before sex diagnosis

Temperature (°C)	Incubated eggs	Hatching success (%)	Duration of incubation (days)	Sex ratio (females/males)
24	27	81.5 (22/27)	88.7 \pm 0.7	12/6
			84.0–93.0	
28	29	96.6 (28/29)	52.8 \pm 0.4	13/11
			51.0–55.0	
30	28	89.3 (25/28)	47.4 \pm 0.3	8/14
			44.0–51.0	
34	50	68.0 (34/50)	39.4 \pm 0.3	13/19
			37.0–42.0	
29.5	24	87.5 (21/24)	51.7 \pm 0.3	11/9
23.7–36.2			48.0–54.0	

Table 2. Influence of incubation temperature on body mass at hatching for *Pelodiscus sinensis*. Initial egg mass is set at 6.0 g when calculating adjusted means. Adjusted means with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$). a > b > c. Only hatchlings of known sex are included

Incubation temperature (°C)	Initial egg mass (g)	Body mass at hatching (g)	
		Observed values	Adjusted values
24	6.05 \pm 0.17	4.28 \pm 0.13	4.24 ^{bc} \pm 0.05
	3.87–6.83	2.68–4.81	3.67–4.50
	5.95 \pm 0.18	4.31 \pm 0.15	4.34 ^{ab} \pm 0.04
28	3.86–6.92	2.65–5.31	4.08–4.74
	5.64 \pm 0.19	4.16 \pm 0.16	4.44 ^a \pm 0.04
30	3.71–6.74	2.66–5.28	4.05–4.78
	6.18 \pm 0.13	4.32 \pm 0.10	4.18 ^c \pm 0.04
34	3.82–6.84	2.66–5.16	3.41–4.55
	6.24 \pm 0.16	4.67 \pm 0.14	4.48 ^a \pm 0.04
23.7–36.2	3.56–6.99	2.44–5.27	4.02–4.75

among temperature treatments. Turtles from the 24 °C treatment were significantly smaller than those from higher incubation temperatures before 1 January 2002 (on average 98 days of age), but the differences in body mass were not significant among turtles from different incubation temperatures on 2 February, 23 March and 23 May 2002 (Fig. 2), when hatchlings from 24 °C

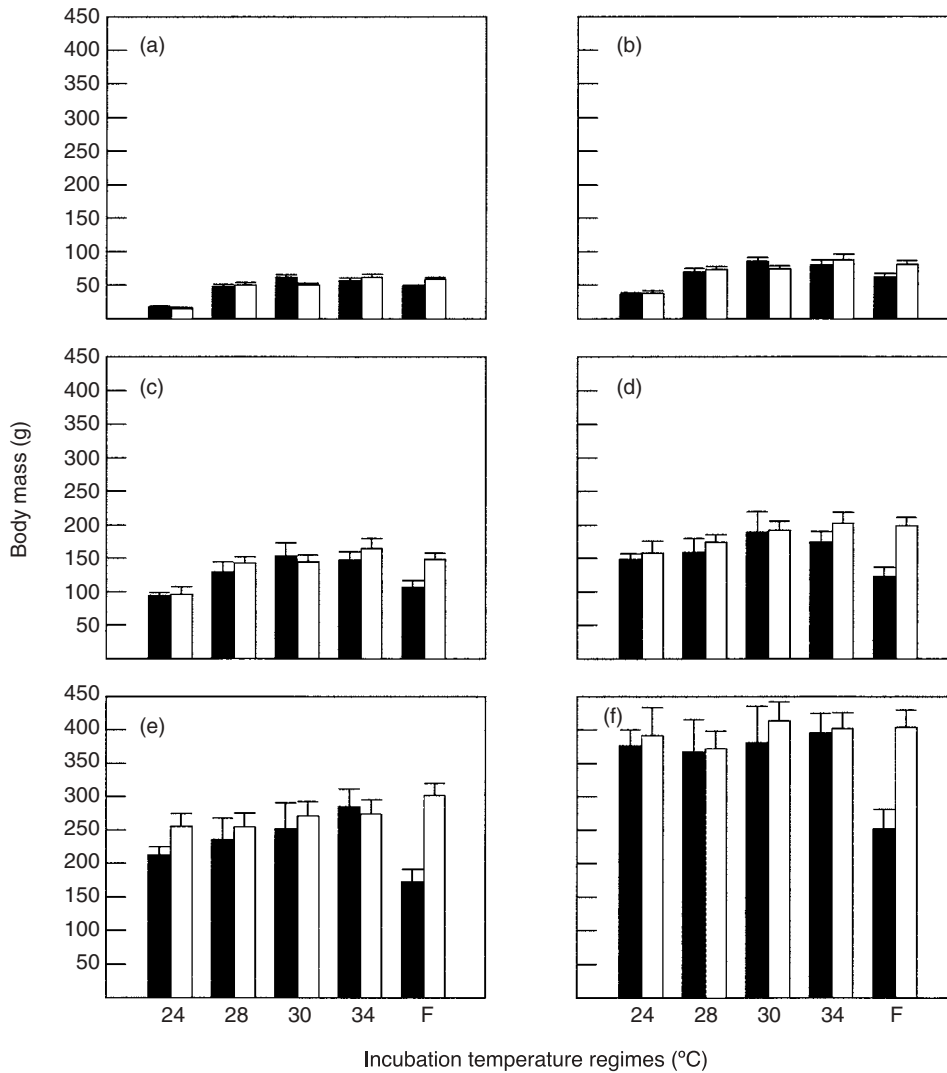


Fig. 2. Post-hatching growth of *Pelodiscus sinensis* hatchlings derived from eggs incubated under different temperature regimes. Values are mean \pm SE. (a) 28 October 2001; (b) 18 November 2001; (c) 1 January 2002; (d) 2 February 2002; (e) 23 March 2002; (f) 23 May 2002. Closed bars, females; open bars, males.

averaged 130, 180 and 240 days of age, respectively (two-way ANOVA, all $P > 0.074$). Overall, male hatchlings grew faster than did females, and became the larger sex from 2 February 2002 (two-way ANOVA, $F_{1,100} = 9.48$, $P < 0.003$). The differences in the overall mean body mass between males and females on 2 February, 23 March and 23 May 2002 were 31.0 (188.4 vs 157.4) g, 39.8 (271.8 vs 232.0) g and 62.5 (397.7 vs 335.2) g, respectively (Fig. 2).

Mass gains of both sexes were positively correlated with the growth period within each temperature treatment ($P < 0.0001$ in all cases). The slopes of the linear regressions of mass gains on growth period differed among turtles from different incubation temperatures (females: $F_{4,24} = 16.18$; males: $F_{4,24} = 5.35$; $P < 0.003$ in both cases) and, in both sexes, turtles from 24°C exhibited either a greater slope or a greater intercept than did turtles from higher incubation temperatures, indicating that turtles from the 24°C treatment grew

faster than did those from higher incubation temperatures. Within the treatments of 24°C ($F_{1,8} = 1.37$, $P = 0.275$), 28°C ($F_{1,10} = 0.08$, $P = 0.782$) and 34°C ($F_{1,10} = 0.08$, $P = 0.778$), the regression slopes did not differ between both sexes. Within the treatments of 30°C ($F_{1,10} = 9.77$, $P = 0.011$) and the fluctuating temperature regime ($F_{1,10} = 42.32$, $P < 0.0001$), the regressions slopes were significantly greater in males than in females (Fig. 3).

DISCUSSION

Lack of TSD in *P. sinensis*

Turtles within Trionychidae are believed to have GSD exclusively, although only a few species in the family have been studied (Vogt & Bull, 1982; Choo & Chou, 1985; Olmo, 1986; Janzen & Paukstis, 1991; but also

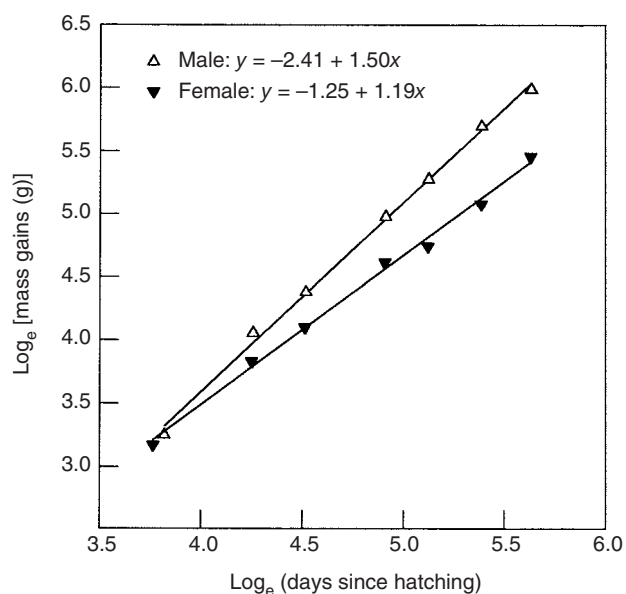


Fig. 3. Linear regressions of mass gain on growth period (days since hatching) for *Pelodiscus sinensis* hatchlings derived from the eggs incubated under fluctuating temperatures. All data are \log_e transformed, and regression equations are indicated in the figure.

see Zhu & Sun, 2000; Nie *et al.*, 2001). Our data do not support a mechanism of TSD in *P. sinensis* because the sex ratio of hatchlings, although slightly biased to females at lower temperatures (24 and 28 °C) and to males at higher temperatures (30 and 34 °C), did not differ significantly from equality within any of the five temperature treatments. The temperatures (from 23 to 35 °C) at which we and other workers incubated *P. sinensis* eggs cover almost the whole range of viable incubation temperatures for the species (Choo & Chou, 1987; Du & Ji, 2001), so it is unlikely that the species has any incubation temperatures which produce exclusively female or male hatchlings.

Zhu & Sun (2000) and Nie *et al.* (2001) considered *P. sinensis* as a TSD species mainly based on two factors: the

lack of heteromorphic sex chromosomes, and highly biased sex ratios at low or high incubation temperatures (Table 3). The lack of heteromorphic sex chromosomes might not be excluded from being a precondition for TSD in the Crocodylia (Janzen & Paukstis, 1991; Lang & Andrews, 1994). However, in other major reptilian taxa, the lack of heteromorphic sex chromosomes is not a necessary condition for TSD, and the presence of heteromorphic sex chromosomes is not always indicative of the absence of TSD (Tokunga, 1985; Deeming & Ferguson, 1991; Janzen & Paukstis, 1991). Thus, any conclusion on sex-determining mechanisms in turtles based on the lack or presence of heteromorphic sex chromosomes alone is problematic. All turtles of the Trionychidae that have been karyotypically examined show morphologically undifferentiated sex chromosomes (Olmo, 1986; Janzen & Paukstis, 1991; Nie *et al.*, 2001), and in the family both *Apalone muticus* and *A. spiniferus* have already been shown to have GSD (Vogt & Bull, 1982; Ewert & Nelson, 1991; Janzen & Paukstis, 1991). Reptiles that have neither heteromorphic sex chromosomes nor TSD also include some species within the Serpentes (Janzen & Paukstis, 1991).

Size at hatching does not affect hatchling growth

Hatchling size has been predicted to be a determinant of fitness in many reptiles as larger sized individuals may have selectively more advantages than individuals of smaller size (Van Damme *et al.*, 1992; Galán, 1996; Braña & Ji, 2000). This prediction probably holds for reptiles living in the wild, where larger young are more successful in hunting prey and avoiding predators (Christian & Tracy, 1981; Avery, Bedford & Newcombe, 1982; P. V. Webb, 1986; Sinervo & Adolph, 1989), have prior access to limited resources, and hence have better chances to survive the first active season and winter (Fox, 1978; Ferguson, Brown & DeMarco, 1982; Ferguson & Fox, 1984; Garland, Hankins & Huey, 1990).

Table 3. The sex ratios of *P. sinensis* hatchlings from different incubation temperatures reported in earlier studies conducted in Singapore and China

Incubation temperature (°C)	Choo & Chou (1985), Singapore			Zhu & Sun (2000), China			Nie <i>et al.</i> (2001), China		
	Incubated eggs	Females	Males	Incubated eggs	Females	Males	Incubated eggs	Females	Males
23	50	11	7	—	—	—	—	—	—
24	—	—	—	60	54	2	110	12	93
25	50	23	8	60	50	5	—	—	—
26	—	—	—	60	47	8	—	—	—
27	—	—	—	60	42	13	116	41	71
28	50	23	23	60	37	20	—	—	—
29	—	—	—	60	27	31	112	59	49
30	—	—	—	60	19	40	—	—	—
31	50	25	16	60	13	46	110	71	37
32	—	—	—	60	7	52	—	—	—
33	—	—	—	—	—	—	118	94	23
34	50	11	9	—	—	—	—	—	—
35	—	—	—	—	—	—	112	98	11

However, size advantages can be substantially masked when reptiles are maintained in the laboratory and other well-designed artificial environments, where resources are usually unlimited, predators are absent and thermal environments are sophisticatedly designed so that an individual's performance at a given temperature can be expressed to an extent much closer to its physiological potential at the corresponding temperature.

In our study, variation in hatchling size resulted mainly from two sources: (1) initial egg mass, which determined size at hatching; (2) influence of incubation temperature, as indicated by the result that eggs incubated at intermediate temperatures produced heavier hatchlings than did eggs at low (24 °C) and high (34 °C) temperatures. However, size at hatching was not a determinant of hatchling growth within each treatment. This finding is contrary to the results reported from short-term hatchling growth studies in lizards in which larger young grew faster than do smaller ones (Van Damme *et al.*, 1992; Galán, 1996; Braña & Ji, 2000). Post-hatching growth is a trait expected to be linked to fitness in reptiles (Brooks *et al.*, 1991; Van Damme *et al.*, 1992; McKnight & Gutzke, 1993; Bobyn & Brooks, 1994; Rhen & Lang, 1995; Braña & Ji, 2000); however, short-term studies may give misleading results about long-term growth. For example, in species with a capacity to compensate its slower initial growth at a later stage, a small hatchling with a high physiological potential can be expected to be small in the early post-hatching days but compensate for its small size during subsequent growth. We are presently unable to evaluate the adaptive significance of body size at hatching for turtles in the wild, because comparable data from the field studies are few (but see Congdon *et al.*, 1999). For *P. sinensis* hatchlings maintained in captivity, size at hatching is at least less important than expected for their long-term growth. Thus, our finding does not provide evidence supporting the 'bigger is better' hypothesis (Congdon *et al.*, 1999), but have an implication for the existence of 'catch-up growth' in small *P. sinensis* hatchlings.

Incubation temperature affects hatchling growth

Post-hatching growth was affected by incubation temperature in our study, with hatchlings from eggs incubated at 24 °C growing faster than did hatchlings from higher incubation temperatures (Fig. 2). This result implies that the temperatures (around 30 °C) at which hatchery operators incubate *P. sinensis* eggs are not optimal for hatchling growth. In this study, eggs incubated at constant temperatures from 28 to 34 °C produced hatchlings that did not differ significantly in post-hatching growth throughout the experiment period, suggesting that the influence of incubation temperature on growth was well buffered within this range.

Phenotypic variation induced by incubation temperatures could be potentially important for reptiles living in the wild, but the extent to which temperature-mediated phenotypic variation has on offspring fitness in nature

remains unknown. For example, in *P. sinensis*, the enhanced hatchling growth through incubating eggs at low temperatures could be balanced by the relatively high embryonic mortality induced by the prolonged exposure time of eggs to the effects of adverse biotic or abiotic factors and the shortened growth period before the onset of the first winter. Thus, under natural conditions, a female's choice of a thermally appropriate nest site can be a way through which she selects optimal thermal environments for embryonic development, so that hatching success, incubation length, post-hatching growth and other offspring phenotypes can be compromised to some extent.

Temperatures in natural nests (particularly shallow nests) vary daily and seasonally, so eggs in the field may potentially experience extreme temperatures during incubation (Chen, Lin & Ji, 2003). Exposure of eggs to low incubation temperatures slows or arrests embryonic development but usually has little or no observable lethal effect on embryos, whereas extremely high temperatures may substantially increase embryonic abnormality and mortality (Sexton & Marion, 1974; Andrews & Rose, 1994; Shine & Harlow, 1996; Andrews, Qualls & Rose, 1997; Ji & Braña, 1999; Ji & Du, 2001a,b; Ji, Du & Xu, 2001; Ji, Qiu *et al.*, 2002). Other important but more underlying adverse effects of extreme incubation temperatures are those on an offspring's performance, including growth. In this study, hatching success at fluctuating temperatures was high and hatchlings were larger than those incubated at 24 °C and 34 °C, indicating that embryos have the ability to tolerate extreme temperatures for brief periods.

The sex effect on growth seemed to be significant only for eggs incubated under fluctuating temperatures because body masses obtained at any of the six post-hatching occasions did not differ between sexes for turtles from the four constant incubation temperatures (ANCOVA with the growth period as the covariate, $P > 0.098$ in all cases). In the fluctuating temperature treatment, males became the larger sex 1 month after hatching and the difference in mass between sexes increased thereafter (ANCOVA with the growth period as the covariate, $P < 0.04$ in all cases). The lower growth rate in female hatchlings suggests an adverse effect of extreme temperatures in the fluctuating temperature treatment on hatchling growth of the sex (Fig. 3). Because the lowest temperature (23.7 °C) in the fluctuating temperature regime was close to the temperature (24 °C) for producing hatchlings with good growth performance, it seemed that the extremely high temperatures experienced by eggs induced this adverse effect. Compared with embryonic females, embryonic males were less vulnerable to extremely high temperatures.

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