

RAPID COMMUNICATION

Temperature-Dependent Sex Determination in the Leopard Gecko, *Eublepharis macularius*

BRIAN E. VIETS, ALAN TOUSIGNANT, MICHAEL A. EWERT, CRAIG E. NELSON, AND DAVID CREWS

Indiana University, Bloomington, Indiana 47405 (B.E.V., M.A.E., C.E.N.);
University of Texas, Austin, Texas (A.T., D.C.)

ABSTRACT The leopard gecko, *Eublepharis macularius*, has temperature-dependent sex determination (TSD). Previous reports have shown that females are produced predominately at cool incubation temperatures and males are produced predominately at warm incubation temperatures (Pattern Ib). We report here that incubation at even higher temperatures (34 and 35°C) produces mostly females (Pattern II). The lethal maximum constant incubation temperature for this species appears to be just above 35°C. Although a previous study indicated that females from a warm incubation temperature (32°C) failed to lay eggs, we found that 12 of 14 mature females incubated at 32.5°C, and 5 of 6 mature females incubated at 34°C produced fertile eggs and viable hatchlings. © 1993 Wiley-Liss, Inc.

Temperature-dependent sex determination (TSD) is a now well-documented phenomenon occurring in all crocodylians thus far investigated (Lang et al., '89), most turtles (Ewert and Nelson, '91), and some lizards (Bull, '83; Tokunaga, '85, '89). *Eublepharis macularius*, the leopard gecko, has been known to have TSD for over a decade (Hofmann, '79; Thorogood and Whimster, '79; Wagner, '80). The previously reported pattern of temperature-dependent sex determination for *E. macularius* (Fig. 1), termed *Pattern Ib* (Ewert and Nelson, '91), has females predominating at cool incubation temperatures (24–28°C) and males predominating at warmer ones (32 and 32.5°C).

Gutzke and Crews ('88) reported that incubation temperature affected not only the sex but also the endocrine physiology and reproductive behavior of adult female *E. macularius*. Adult females incubated at 26°C (an exclusively female-producing temperature) and 29°C (a mostly female-producing temperature) differed both hormonally and behaviorally from females incubated at 32°C (a mostly male-producing temperature). Radioimmunoassay (RIA) of circulating concentrations of sex steroid hormones showed that androgen levels were highest, and estradiol levels lowest, in females that had experienced an incubation temperature that produces mostly males (32°C). In addition, these 32°C females were more likely to exhibit aggressive behavior toward either sex, whether in their home cage or a neutral site. The 32°C females responded to courtship by males as if they themselves were male, and none of these females laid eggs during the study

(2 years, N = 6). Gutzke and Crews ('88) suggested that these 32°C females were functionally sterile. Because the reproductive fitness of the 2 sexes was differentially affected by incubation temperature, their findings were consistent with the Charnov-Bull model (Charnov and Bull, '77) for the evolution of environmental sex determination, of which TSD is one form.

Here, applying an approach of incubating reptilian eggs across the full range of viable temperatures for the species (Ewert and Nelson, '91), we report a different sex-determining pattern for *E. macularius*, as well as reproductive data for high temperature (32–35°C) females.

MATERIALS AND METHODS

Geckos were housed in environmentally controlled chambers in breeding pairs or trios (Indiana University [IU]), or in groups with one male and 4–20 females (University of Texas [UT]). The lizards were fed crickets, mealworms, and neonatal mice. Food supplements were added, including Vionate® (Rich Health) and calcium carbonate at IU; and vitamin-mineral powder (Petco Animal Supplies), pulverized chelated magnesium (Kal, Inc.), and pulverized time-released vitamin B-1 (Nature-Made Nutritional Products) at UT. Water was provided ad libitum.

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B.E. Viets' present address is Biology Department, Nebraska Wesleyan University, 5000 St. Paul Ave, Lincoln, NE 68504.

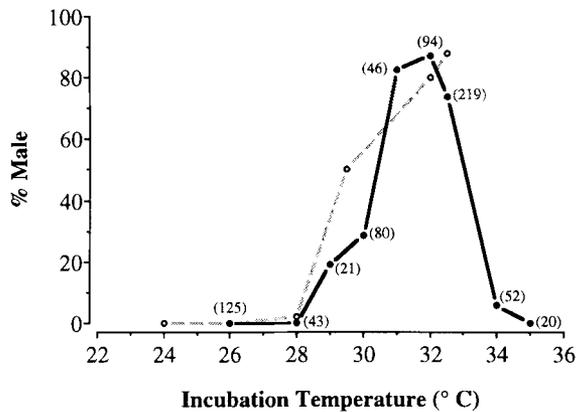


Fig. 1. Hatchling sex ratio (% male) as a function of various constant incubation temperatures in *Eublepharis macularius*. The previously known sex-determining pattern is indicated by the dashed line and open circles (data are from Bull '87a,b; Wagner '80). Data from this study are shown by the black line and solid circles, with sample sizes indicated in parentheses. Incubation temperatures with a sample of less than 20 were omitted (but see Table 1).

Eggs were obtained from matings in the laboratory during 1989–1991. Gravid females were provided with nesting areas of moist vermiculite (IU) or sand (UT). Cages were checked at least once daily for eggs. Eggs were candled to check for fertility and were allocated to an incubation temperature. Each clutch (normally 2 eggs) was usually split between incubation temperatures.

Although different incubation techniques were utilized at the 2 institutions, the end results are very similar. Most eggs were incubated in 1.5:1 water:vermiculite by mass, but some were incubated using 1:1 or 2:1 moisture regimes. However, the hydric content of the substrate does not affect the sex ratio in this species (B. Viets and L. Talent, unpublished data).

Eggs were placed in airtight plastic Rubbermaid® boxes (38 × 26 × 13 cm) and flushed with air daily (IU), or were placed individually in plastic cups with perforated tops (UT). The incubation boxes were placed in incubators and rotated daily to eliminate possible temperature gradient effects. Temperatures in the upright, cabinet-type incubators very seldom varied more than $\pm 0.2^{\circ}\text{C}$ from the design temperature during the course of the study.

Eggs allocated to temperatures from 26 to 33°C were placed at the design temperature on the day of oviposition and remained there until hatching or death (Table 1). Eggs destined for 34°C spent the first day at 32°C, were shifted to 34°C for the bulk of the incubation period, and were shifted back to 32°C for 1–3 days before hatching. At 35°C, 12 eggs

were shifted in the same fashion as the 34°C eggs, and 8 eggs were placed immediately at 35°C and remained there until hatching or death. *E. macularius* eggs are usually at stage 30 of Dufaure and Hubert ('61) when laid, although they range from stage 29 to 31 (Bull, '87a; our observations). As the period of irreversible sex determination in this species lies between stages 32 and 37 (Bull, '87a), these shifts should not have affected sex determination. In fact, 91% of the total incubation time at 34°C (mean 34.7 days, SD = 0.83) and 92% of the total incubation time at 35°C (mean 35.9 days, SD = 2.97) was spent at the design temperature. This shifting to a cooler temperature at the end of incubation is important; all 12 of the 35°C shifted eggs hatched, whereas only 2 of 8 of the nonshifted eggs hatched.

The sex of the hatchlings was diagnosed by the presence of secondary sexual characteristics—males have large, preanal pores with plugs and, usually, a hemipenial bulge; females have much smaller pores with no plugs. Sex was confirmed by dissection on all animals that died during the course of the study. In addition, laparotomies were performed at UT when sex diagnosis was required at an early age, using criteria developed by Bull ('87b). To date, there has been total agreement of sex diagnosis by all methods employed.

RESULTS

Sex-determining pattern

Sex ratios for *E. macularius* differed markedly among incubation temperatures (chi-square, $P \leq 0.0001$; Fig. 1). Sex ratios at common incubation temperatures (26, 30, and 35°C) did not differ significantly between institutions (ANOVA, $P = 0.40$; Table 1). Females were produced exclusively (@ 26 and 28°C) or predominately (81% @ 29°C; 71% @ 30°C) at cool incubation temperatures. Males predominated at intermediate (31–33°C) temperatures, with 31.5°C producing the most males (88.9%). At warmer temperatures, females were again produced predominately (94% @ 34°C) or exclusively (@ 35°C). A pattern of sex determination that produces females at cool and warm temperatures, and males at intermediate temperatures, was termed *Pattern II* by Ewert and Nelson ('91).

Egg production by high-temperature females

Twelve of 14 females incubated at 32.5°C and currently of reproductive age have produced 35 viable offspring. In addition, five of the six 34°C females currently of reproductive age have produced viable offspring (39 fertile eggs, 20 viable offspring, 19 fer-

TABLE 1. Effect of different constant incubation temperatures on hatching success and sex ratio in *Eublepharis macularius*

°C	Indiana University				University of Texas			
	NFE	%H	NS	%M	NFE	%H	NS	%M
26	5	100	5	0	207	58.0	120	0
28	44	95.5	43 ¹	0	—	—	—	—
29	1	100	1	0	—	—	20 ⁴	20.0 ⁴
30	40	97.5	39	33.3	66	62.1	41	24.4
31	—	—	—	—	—	—	46 ⁴	82.6 ⁴
31.5	—	—	—	—	14	64.3	9	88.9
32	95	98.9	94	87.2	—	—	—	—
32.5	—	—	—	—	347	63.1	219	74.0
33	5	100	5	80.0	—	—	—	—
34	52	96.2	52 ²	5.8	—	—	—	—
35	14	85.7	12	0	10	20.0	8 ³	0
Totals	256	96.9	251	—	644	60.7	463	—

¹Includes 1 sexable, full-term embryo.

²Includes 2 sexable, full-term embryos.

³Includes 6 sexable, full-term embryos.

⁴Unpublished data from J.J. Bull. Not included in the calculation of hatching success.

°C = incubation temperature; NFE = number of fertile eggs incubated; %H = hatching success; NS = number of hatchlings or term embryos for which sex was determined; %M = sex ratio (expressed as percent male).

tile eggs still incubating). In each case, our mature females produced eggs and viable offspring at their first reproductive opportunity.

Other laboratories have also had females from warm incubation temperatures reproduce successfully. At Oklahoma State University, 10 of 10 mature 32°C females and 5 of 6 mature 35°C females have produced viable offspring (L. Talent, personal communication). At Memphis State University, 5 of 8 mature 35°C females have produced viable offspring (W. Gutzke, personal communication).

An upper thermal limit to sustained development

Incubation temperature significantly influenced the duration of incubation in *E. macularius* (ANOVA, $P = 0.001$). Incubation temperature and mean days to hatching were inversely correlated from 26°C (72.2 days, SD = 4.256) to 32.5°C (35.9 days, SD = 1.761). Mean developmental rates (when transformed to days to hatching⁻¹ × 1000) are colinear from 26 to 32.5°C (Fig. 2). However, the rate for 34°C is distinctly slower than would have been expected, and the rate for 35°C is even slower. Because hatching success at 35°C was greatly reduced in unshifted eggs, and because the observed mean days to hatching was slower than expected (and even slower than at 34°C), the lethal maximum constant incubation temperature for this species probably lies just above 35°C.

Hatching success

Hatching success differed significantly between the 2 institutions (chi-square, $P \leq 0.0001$). However, at each institution hatching success did not differ significantly among temperatures from 26 through 34°C ($P = 0.73$ @ IU; $P = 0.68$ @ UT). Hatching success at 35°C was markedly (but not significantly)

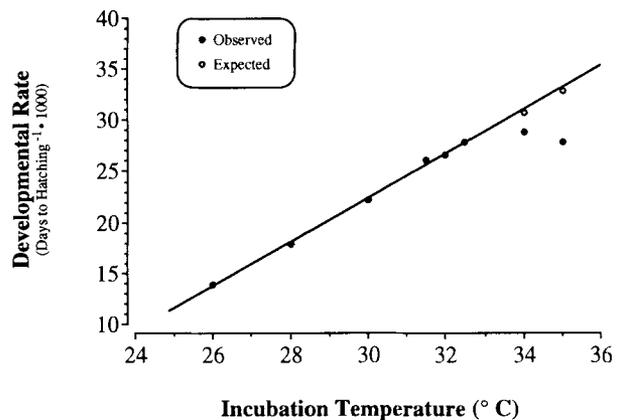


Fig. 2. Mean developmental rate (as the reciprocal of total incubation time × 1000) at various constant incubation temperatures for *Eublepharis macularius*. Observed values for 34 and 35°C are based only on the data for shifted eggs (see text). Expected values for 34 and 35°C were calculated using the regression equation for data from 26–32.5°C ($y = -42.856 + 2.1752x$; $R^2 = 0.998$, $P < 0.0001$) and taking the temperature shifts for 34 and 35°C into consideration. Raw data were transformed to achieve colinearity of points across most of the temperature range.

lower at both institutions ($P = 0.19 @ IU$; $P = 0.07 @ UT$). Overall, of 900 fertile eggs tallied and monitored, 639 (71.0%) hatched (Table 1).

DISCUSSION

Sex-determining pattern

Ewert and Nelson ('91) noted that the determination of the pattern of TSD often requires an examination of most of the range of viable incubation temperatures for the species. Previous studies (Bull, '87a,b; Wagner, '80) of the sex-determining pattern of *E. macularius* found a cool-female, warm-male type pattern (Pattern Ib of Ewert and Nelson, '91) but utilized only part of the viable range of incubation temperatures. Our study of temperatures warmer than those previously examined shows that the range of viable incubation temperatures for *E. macularius* extends from 24 to 35°C (Fig. 1). Females again predominate at warm incubation temperatures, indicative of Pattern II TSD rather than Pattern Ib TSD.

Given that 34 and 35°C may be viewed as stressful, differential embryonic mortality might be suspected (contrary to Bull, '87b) as a cause of sex ratio skews in eggs of *E. macularius* incubated at very warm temperatures. However, the data from IU rule out differential mortality: embryos from all 52 eggs at 34°C survived to be diagnosed, and only 2 of 14 at 35°C were lost. Even if each of these 2 individuals were assigned to the rarer sex, the effect on the observed sex ratios would be minimal.

Developmental rate and total incubation time have been proposed as better predictors of offspring sex ratio than incubation temperature (Webb and Smith, '84; Webb et al., '87). Because the rate of development at 34 and 35°C was slowed and total incubation time was longer relative to slightly cooler incubation temperatures, it is possible that these parameters, not incubation temperature, are the ultimate determinants of sex. However, developmental rates at 32 and 35°C were very similar (Fig. 2), but they yielded 74.0% and 0% males, respectively. In addition, a series of experiments which manipulated developmental rate and total incubation time independent of incubation temperature in *Trachemys scripta* failed to confirm Webb and Smith's ('84) and Webb et al.'s ('87) hypothesis (Etchberger et al., 1992). Similar experiments with the eggs of *E. macularius* would likely yield similar results.

Pattern II has already been established for another gecko (*Gekko japonicus*; Tokunaga, '85, '89). In fact, Pattern II is the only pattern of TSD common to the 3 major reptilian lineages that have TSD (Ewert

and Nelson, '91) and may be the primitive state in reptiles (Deeming and Ferguson, '89).

Eublepharis macularius breeds in the hot season (Daniel, '83), and soil temperatures during times of activity range from 30.2 to 35°C (Anderson, '63). Thus, high-temperature females (34 and 35°C) may well be produced in the wild. At the least, occasional females should be produced at fairly high incubation temperatures (31–33°C), as no known constant temperature produces 100% males in this species (Table 1; Fig. 1). In addition, warm-temperature females are produced in the field in some other Pattern II reptiles (*Crocodylus palustris*, Lang et al., '89; *C. porosus*, Webb and Cooper-Preston, '89; *C. johnstoni*, Webb and Smith, '84).

Behavioral and physiological implications

With the discovery of Pattern II TSD in *E. macularius*, there now exist females produced at incubation temperatures (34 and 35°C) warmer than those that produced the "hot females" (32°C) of Gutzke and Crews ('88). Most (15 of 20) of our mature 34 and 35°C females have already mated and produced viable hatchlings. In addition, most (22 of 24) mature females produced at mostly male-producing temperatures (32 and 32.5°C) have produced viable hatchlings, although the 32.5°C females tend to exhibit heterotypical behaviors more often than cooler temperature females (D. Flores, A. Tousignant, and D. Crews, unpublished data). It is not currently known whether the 32°C and warmer females are receptive, or whether copulation is being forced by the larger male. Gutzke and Crews ('88) and ongoing studies (A. Tousignant, B. Viets, and D. Crews, unpublished data) indicate that females produced at mostly male-producing temperatures (32 and 32.5°C) differ hormonally and behaviorally from females incubated at mostly female-producing temperatures (26 and 28°C, and 34 and 35°C). The differences observed in the reproductive success of "hot females" between this study and the study of Gutzke and Crews ('88) may be due to differences in housing conditions. When females are paired with males in relation to the onset of vitellogenesis is critical to subsequent ovulation (A. Tousignant and D. Crews, unpublished data). At present, however, there appear to be no differences in reproductive success between high-temperature (32, 32.5, 34, and 35°C) and low-temperature (26 and 28°C) females.

Eublepharis macularius has been used to support the Charnov-Bull model for the evolution of environmental sex determination, based on the apparent functional sterility of females produced

at a warm, mostly male-producing temperature (32°C). However, our study found no qualitative differences in the reproductive success of females produced at warm to very warm incubation temperatures (32–35°C). Thus, subtler criteria will be needed (e.g., behavior, relative fertility) in order to assess the applicability of the Charnov-Bull model to this species. Thus far, strong evidence for the adaptive nature of the different sex-determining mechanisms that occur in reptiles remains elusive.

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