Ecology, 82(11), 2001, pp. 3010–3024

CONSTANT, SHIFT, AND NATURAL TEMPERATURE EFFECTS ON SEX DETERMINATION IN *PODOCNEMIS EXPANSA* TURTLES

NICOLE VALENZUELA[1]

Department of Ecology and Evolution, State University of New York, Stony Brook, New York 11794 USA, and Fundación Puerto Rastrojo, Carrera 10 Número 24-76, Oficina 1201, Santafé de Bogotá D.C., Colombia

Abstract. Environmental sex determination may yield biased sex ratios, as is the case in many reptilian species with temperature-dependent sex determination (TSD). Determining TSD thermal ecology is important to understanding sex ratio evolution in those species, as TSD systems may pose a challenge to Fisherian models that predict the production of 1:1 sex ratios through frequency-dependent selection. Unfortunately, the relationship between fluctuating temperatures and sex in nature remains poorly understood. Here I present a novel statistical model of TSD in the endangered Amazonian freshwater turtle, *Podocnemis expansa,* designed to account for heterogeneous daily fluctuations of natural nest temperatures by comparing laboratory and field data on sex ratios, developmental stages, cumulative temperature units (CTUs), and time spent below the survival threshold (28°C). Experiments encompassed constant, shift-once, and shift-twice temperatures in the laboratory, plus depth and shading treatments in the field. A constant temperature of 32.6°C produced 1:1 males : females, the highest pivotal temperature ever reported for a turtle species. The thermosensitive period for sex determination (TSP) roughly occurs during developmental stages 17–24 as determined from shift experiments. The CTUs during the TSP plus the number of hours below 28°C prior to the TSP significantly explained sex ratio production in the combined laboratory and field data set (positive and negative effects, respectively). However, the relationship for field nests only was not significant. Higher temperatures had a disproportionately larger effect on female production in the combined field and laboratory data set. Depth and shading had drastic effects on nest temperature, embryonic development, and sex ratio. Seasonal climate changes can override the effect of nest depth via differences in oviposition time. Two of the shaded nests experienced the coldest temperatures of all field nests but yielded the highest female proportion, according to a TSDII pattern (when females are produced at low and high temperatures and males are produced at intermediate temperatures). Thus *Podocnemis expansa* may be a TSDII species, but this intrinsic system seems to be realized as TSDIa in nature (males produced at low temperatures and females produced at high temperatures), because of the general lethality of cold temperatures which precludes the production of low-temperature females.

Key words: Amazonia; endangered species; pivotal temperature; Podocnemis expansa; *reptiles; temperature-dependent sex determination (TSD); thermosensitive period (TSP); turtles; sex determination.*

INTRODUCTION

Theoretical models of sex ratio evolution are concerned with the proportion of the sexes and the mechanisms behind the allocation of resources to males and females (Karlin and Lessard 1986, Bulmer 1994). Even sex ratios (1:1) are predicted to evolve through frequency-dependent selection (Darwin 1871, Fisher 1930). Most empirical data are consistent with this expectation although there are several notable exceptions (Karlin and Lessard 1986, Bulmer 1994, and references therein). Some of these exceptions are adaptive modifications of offspring sex ratio by females as in the case of haplodiploid organisms (e.g., Nonacs 1986, Boomsma 1989), situations of local mate–resource competition (e.g., Hamilton 1967, Clark 1978, Johnson 1988), and instances where fitness varies with the environment (e.g., Trivers and Willard 1973, Charnov 1982, Clutton-Brock et al. 1984) as in the case of organisms with temperature-dependent sex determination (TSD) (e.g., Bull 1983, Conover 1984). Sex ratios, like other life history components, affect population parameters directly (Roff 1992, Stearns 1992). Consequently, it is important to understand mechanisms like TSD that allow the production of biased sex ratios.

In reptiles, genotypic and temperature-dependent sex determination co-occur (Bull 1980, Ewert and Nelson 1991, Janzen and Paukstis 1991, Ewert et al. 1994, Viets et al. 1994, Lang and Andrews 1994, Wibbels et al. 1994). In TSD, sex is determined after fertilization by the incubation temperature that the embryo experiences during a limited window of time, called the thermosensitive period (Bull 1983, Mrosovsky and

Manuscript received 20 January 2000; revised 13 November 2000; accepted 20 November 2000.

[1] Present address: Department of Zoology and Genetics, Iowa State University, 339 Science II, Ames, Iowa 50011-3223 USA. E-mail: nvalenzu@iastate.edu

Pieau 1991). Turtles exhibit two modes of TSD; TSDIa in which females are produced at high temperatures and males at low ones, and TSDII in which females are produced at high and low temperatures and males at intermediate ones (Ewert and Nelson 1991, Ewert et al. 1994). A third mode, TSDIb (the reciprocal of TSDIa), was originally reported in lizards and alligatorines; however the evidence for some species has been contested (Ewert et al. 1994, Viets et al. 1994, Lang and Andrews 1994).

Studying the thermal ecology of TSD systems is crucial if we wish to understand sex ratio evolution in TSD species. Unfortunately, the relationship between fluctuating temperatures in natural nests and offspring sex ratio remains obscure. Mean incubation temperature is a poor predictor of field sex ratios for some TSD species, including the endangered Amazonian freshwater turtle *Podocnemis expansa* (Pieau 1982, Schwarzkopf and Brooks 1985, Georges 1989, 1992, Valenzuela et al. 1997). Alternative models to the use of mean incubation temperature have not been generally applicable. Namely, models incorporating the mean and variance of nest temperature (Bull 1985, Souza and Vogt 1994), the number of hours at or above the temperature that produces 1:1 sex ratios during the thermosensitive period (Pieau 1982, Wilhoft et al. 1983, Bull 1985, Schwarzkopf and Brooks 1985, Mrosovsky and Provancha 1992, Souza and Vogt 1994), or a model of fluctuating temperature (Georges 1989), were applicable to only some of those species. They were all poor predictors for *Podocnemis expansa* (Valenzuela et al. 1997). The number of hours above 31°C and mean temperature above 31°C during day 29 or 30 of incubation were the two parameters that best explained sex ratios in a previous field study of *P. expansa* (Valenzuela et al. 1997), but the biological significance of such a short period of time is still uncertain. Determining the thermal mechanism of TSD in nature is particularly important for endangered species that, like *P. expansa,* require the prompt evaluation of current management strategies including nest transplants and seminatural incubation programs (von Hildebrand et al. 1997).

Embryonic developmental rate, temperature, and sex should be considered simultaneously when studying TSD (Pieau 1982, Webb et al. 1987, Webb and Cooper-Preston 1989) to account for variation in the timing of the thermosensitive period (TSP). Additionally, nest temperature should be monitored continuously over the entire incubation period (e.g., Georges 1989, Mrosovsky and Provancha 1989) to account for seasonal thermal variation. The duration and magnitude of temperature exposure during the TSP has a cumulative effect on sex differentiation of TSD species (Johnston et al. 1995). Moreover, this cumulative effect varies with the amplitude of the temperature fluctuation (Georges 1989).

The ecological, and evolutionary, significance of TSD can only be appreciated if we understand the influence of temperature on sex ratio in natural nests (Georges 1992). Only with this knowledge can we unravel the proximate mechanisms, the magnitude, and the ecological context of the sex ratio fluctuations that populations of TSD species undergo in the short and long term. The present study was designed to explore the effects of variable thermal regimes on TSD in order to characterize the thermal mechanism of sex determination in *P. expansa,* while deriving a statistical model that may account for the effects of heterogeneous daily fluctuations of temperature experienced by nests in the field. Laboratory and field data on developmental stages and cumulative temperature exposure over time were compared to accomplish this goal.

Methods

Study site

The study was carried out in the Middle Caquetá River, Colombian Amazonia, at the Tamanco region, which comprises the nesting beaches of Tamanco and Playa Roja (0°50.5′ S, 71°48.7′ W). *Podocnemis expansa* females nest on sandbars that emerge as the river level lowers during the dry season (September to February). Average clutch size in the study area is 103 eggs (50–184) (von Hildebrand et al. 1997, Valenzuela 2001). Clutches collected from both beaches were used in experimental nests, and some clutches were transported to the Colombian capital, Santafé de Bogotá, to perform the artificial incubation experiments. This study was conducted during a single reproductive season between October 1997 and February 1998.

Artificial incubation

Eggs were placed in 25 × 25 × 6 cm trays and buried in a mixture of vermiculite, sand from a nesting beach, and water in equal proportions. Trays containing only the incubation substrate were weighed at the onset of the experiments and every week after that to estimate water evaporation. Water was added to the substrate every week if any loss was detected to maintain a relatively constant moisture level in all incubators. Trays were shifted weekly within incubators and eggs were rotated within trays to control for possible temperature clines. The same methodology was used in the shift experiments. One egg per treatment was sampled weekly to establish the developmental curve at each constant temperature and shift-once experiment. (Limited egg numbers available for the shift-twice experiments precluded this weekly sampling.) These embryos were not sexed. Hatchlings were sexed by radioimmunoassay of testosterone levels in the blood, a method that gives information on sex for this species (Lance et al. 1994, Valenzuela et al. 1997).

Constant temperature.—Eggs from three clutches were uniformly distributed among four electric incubators (30 eggs per incubator) set at 30.5°C, 32.5°C,

TABLE 1. Mortality and sex ratios of *Podocnemis expansa* from all laboratory experiments and field nests.

Treatment (nest ID)	Eggs per treatment† (eggs sampled)‡	Eggs hatched	Hatchlings sexed	Mortality after TSP onset (%)§	Total embryo mortality (%)‖	Sex ratio (% females)
Field treatment						
15 cm Shade (14)	87 (11)	0	···	51.3	100	···
15 cm Shade (3)	100 (13)	7	6	66.7	92	83.3
15 cm Shade (16)	107 (12)	0	···	42.1	100	···
15 cm Shade (12)	92 (14)	1	1	76.9	98.7	0
30 cm Shade (2)	99 (14)	0	···	47.1	100	···
30 cm Shade (6)	108 (13)	29	15	55.8	68.8	73.3
30 cm Shade (13)	105 (13)	0	···	53.3	100	···
45 cm Shade (11)	93 (14)	0	···	29.1	100	···
45 cm Shade (21)	100 (12)	0	···	22.7	100	···
15 cm Sun (9)	100 (9)	35	32	31.9	61.5	28.1
15 cm Sun (22)	101 (9)	53	30	30.4	42.4	26.7
15 cm Sun (19)	86 (9)	53	31	15.6	31.2	29.0
15 cm Sun (17)	96 (9)	62	29	11.5	28.7	20.7
30 cm Sun (7)	101 (9)	62	29	14.1	32.6	41.4
30 cm Sun (4)	98 (10)	32	31	17.0	64.8	58.1
Natural 30 cm (8)	87 (9)	33	30	9.0	57.7	46.7
Natural 30 cm (18)	105 (8)	91	30	3.1	6.2	6.7
45 cm Sun (5)	98 (8)	56	30	4.4	36.4	13.3
45 cm Sun (10)	98 (9)	42	32	10.1	52.8	25
Natural 45 cm (15)	86 (8)	60	30	11.5	23.1	60
Laboratory treatment						
Constant 30.5°C	32 (8)	22	20	12.5	16.7	10
Constant 32.5°C	55 (8)	12	10	51.1	70.2	70
Constant 33.5°C	75 (7)	12	11	69.1	75	81.8
Constant 34.5°C	35 (6)	13	13	48.3	48.3	92.3
Shift 30-34 day 14	15 (4)	4	3	22.6¶	54.5	66.7
Shift 30-34 day 21	14 (3)	9	7	22.6¶	27.3	100
Shift 30-34 day 28	13 (3)	3	3	22.6¶	40	100
Shift 30-34 day 35	12 (2)	8	7	22.6¶	10	57.1
Shift 30-34 day 42	11 (1)	6	6	22.6¶	20	50
Shift 34-30 day 14	15 (5)	4	4	33.6¶	50	0
Shift 34-30 day 21	14 (4)	7	7	33.6¶	30	42.9
Shift 34-30 day 28	13 (2)	8	7	33.6¶	9.1	42.9
Shift 34-30 day 35	12 (2)	7	6	33.6¶	10	66.7
Shift 34-30 day 42	11 (1)	9	8	33.6¶	20	75
Shift 34-30-34 day 21	14 (2)	6	5	5.6¶	58.3	80
Shift 34-30-34 day 28	13 (3)	9	7	5.6¶	0	42.9
Shift 34-30-34 day 35	7 (0)	6	6	5.6¶	0	66.7
Shift 34-30-34 day 42	7 (0)	8	7	5.6¶	0	14.3
Shift 30-34-30 day 21	15 (3)	10	8	8.3¶	33.3	87.5
Shift 30-34-30 day 28	12 (2)	10	8	8.3¶	20	50
Shift 30-34-30 day 35	10 (0)	7	7	8.3¶	30	100
Shift 30-34-30 day 42	10 (0)	10	10	8.3¶	0	80

Notes: For explanation of all treatments see *Methods: Field incubation*, and *Artificial incubation*. All temperatures in the shift experiment are given in °C. Mortality precluded the transfer of the anticipated number of eggs as planned for the shift-twice experiments.

† Eggs per treatment = total initial number of eggs.

‡ Eggs sampled = number of eggs taken for embryological series out of the total initial number.

§ Mortality after TSP onset = embryos that died after developmental stage 17 and before hatching (field), including embryos whose developmental stage at death was not known (laboratory).

‖ Total embryo mortality = dead embryos out of total initial number of eggs minus the eggs sampled or broken during handling.

¶ Mortality values for the entire shift treatment are reported irrespective of transfer day.

33.5°C, and 34.5°C (±0.5°C), to control for clutch effects on sex ratio. Extra eggs were distributed unevenly in the four incubators such that final sample sizes differed among temperatures (Table 1). The basic pattern of temperature effects on sex ratios and the pivotal temperature for this turtle species were determined from these data.

Shift-once experiment.—A group of 65 eggs from the same three clutches was placed in each of two incubators set at 30.5°C and 34.5°C. These temperatures

were expected to yield ~100% males and ~100% females, respectively (Lance et al. 1994). At weeks 2, 3, 4, 5, and 6 of incubation a subgroup of eggs was moved from 30.5°C to 34.5°C and kept there until hatching (15, 14, 13, 12, and 11 eggs, respectively); the reciprocal shift was also done from 34.5°C to 30.5°C.

Shift-twice experiments.—Eggs from two additional clutches were distributed uniformly into two incubators (77 eggs per incubator) set at 30.5°C and 34.5°C. On the second week of incubation, all the eggs were transferred from 30.5°C to 34.5°C. The reciprocal shift was performed as well. At weeks 3, 4, 5, and 6 of incubation 14, 13, 12, and 11 eggs, respectively, were taken back to their original temperature and kept there until hatching. This pattern of shifts exposed embryos to a pulse of female-producing (34.5°C) or male-producing (30.5°C) temperatures for variable periods of time (see Fig. 1).

Field incubation

Seminatural incubation.—A total of 17 experimental nests were buried in a nesting beach at three depths, 15, 30, and 45 cm from the surface to the first egg. Each seminatural egg chamber was 20 cm deep such that total depth was 35, 50, and 65 cm, respectively. Experimental nests consisted of groups of ~100 eggs placed inside a plastic mesh bag. The material of the bags allowed water drainage and gas exchange, while reducing attacks by predatory crickets (*Grillotelpidae*) and preventing the mixing of emerging hatchlings from different nests. Eggs from two to seven clutches from the same oviposition night were distributed uniformly among experimental nests to control for clutch effects. Experiments were set in different days in order to ensure the production of varied sex ratios by breaking up the developmental synchrony introduced when oviposition dates are homogeneous (Valenzuela et al. 1997). Nests were buried at the nesting beach in two 3 × 3 m plots, one shaded by a 1-m-high 3 × 3 m palm leaf roof, and one uncovered. In each plot depth treatments were assigned according to a Latin square design (Sokal and Rohlf 1995). The number of nests per depth and shading treatment was not balanced (Table 1). The depth treatments were expected to produce different temperature conditions that would simulate natural conditions from cloudier or sunnier seasons or from nests laid by females of different sizes (Valenzuela 2001). For example, temperatures may be lower and may fluctuate less in deeper nests than in superficial nests.

Natural incubation.—As a control group, three natural nests were monitored where they were laid, without mesh bags. In all seminatural and natural nests, temperature was recorded every hour with an Onset Optic StowAway Temp datalogger (Onset Computer, Pocasset, Massachusetts, USA) buried at mid-depth within the egg chamber. Readings of percent water content of sand for each field nest were taken once or twice

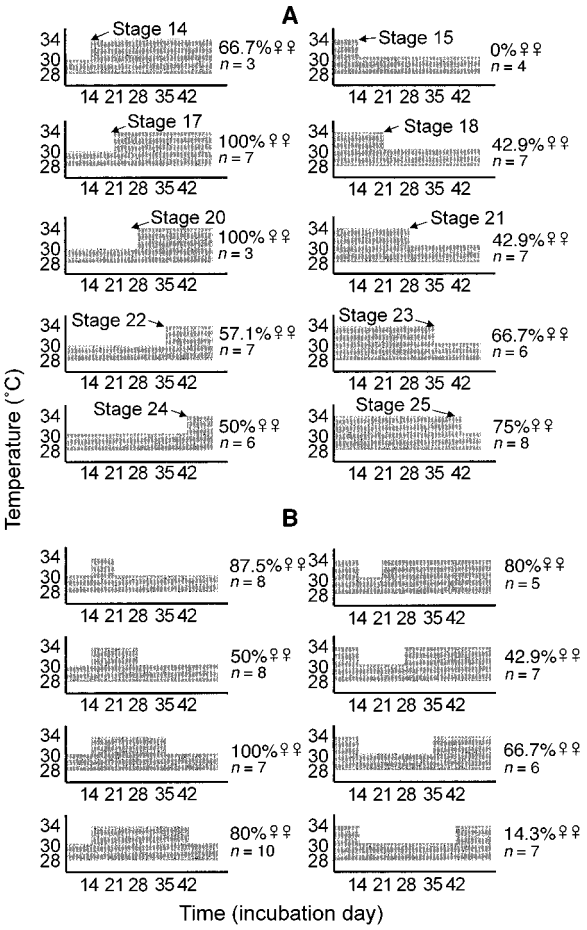

FIG. 1. *Podocnemis expansa* sex ratios resulting from the shift-temperature experiments: (A) shift-once and (B) shift-twice experiments. Cumulative temperature units (CTUs) are depicted by the shaded area under the temperature trace above the developmental threshold of 28°C (Valenzuela et al. 1997). The CTUs are a modification of the degree-days concept as used by Schwarzkopf and Brooks (1985).

daily using a carbonate block buried at mid-depth adjacent to the egg chamber, and connected to a Beckman Soil Moisture Bridge (Beckman Instruments, Allendale, New Jersey, USA). Hatchlings were collected right before emergence. One egg was sampled weekly from each nest to determine developmental stage. Thirty hatchlings (or as many as were available if <30) were chosen at random from each nest to determine sex by radioimmunoassay.

Data analysis

Cumulative temperature units (CTUs) in each treatment were calculated as the area below the hourly temperature curve and above the survival threshold of 28°C (Fig. 1). This was done by subtracting 28 from each temperature record that exceeded this value, and then adding up the resulting values. The CTUs generated in this study reflect both the duration and magnitude of temperature exposure described by Johnston et al.

(1995). The CTUs for the entire incubation period or for particular developmental periods (e.g., for the thermosensitive period) were calculated for different analyses and will be mentioned in each case.

Regression analysis was used to calculate the influence of CTUs and of temperatures below 28°C on developmental rate. This threshold value was chosen because a previous study demonstrated that constant temperatures of 28.5°C or below were lethal for this species (Valenzuela et al. 1997). Logistic regression (Sokal and Rohlf 1995) was used to test the effect of those two temperature variables on mortality. A maximum likelihood method (Girondot 1999) was used to calculate the pivotal temperature using data about the number of males and females produced at each constant incubation temperature during this study as well as previous data (Lance et al. 1994, Valenzuela et al. 1997). The pivotal temperature and its 95% confidence limits were also calculated using inverse prediction from the logistic regression of sex ratios on constant temperature using JMP (SAS 1995).

From shift-once experiments the TSP was determined in terms of chronological days (after which no changes in sex ratios were produced by the shifts) and in terms of developmental stages. The CTUs accumulated during the period of time comprising those developmental stages were calculated for each artificial incubation condition. The shift-twice design allowed for the accumulation of different numbers of CTUs per treatment (duration at the second temperature) (Fig. 1). A logistic regression model (Sokal and Rohlf 1995) was used to find the association between sex ratio and temperature variables. Initially, data from constant incubation temperature and shift experiments were included in the logistic regression analysis of CTUs and sex ratios. Subsequently, CTUs for field nests were calculated for the same developmental period as for laboratory experiments. The field CTUs were added to the laboratory data and the logistic regression was recalculated. An analysis of covariance was performed to determine if there was a significant interaction of CTUs and nest origin (field and laboratory) on sex ratio in order to test if the relationship of sex and temperature from the field was consistent with the laboratory. Finally, the number of hours spent below 28°C was calculated for the field nests and this variable was included with the CTUs in a multiple logistic regression model. This temperature parameter was used because sex determination in natural nests is related to developmental rate (Georges 1989), and the time of exposure to temperatures below the survival threshold may retard development.

Statistical analyses were performed using BIOMstat 3.2c (Rohlf and Slice 1999) and JMP 3.0 (SAS 1995) software packages. The implicit assumption that there is a linear relationship between temperature and development was tested directly by polynomial regression using the developmental series and CTU data from laboratory experiments and field nests. Effects of field experimental treatments on several nest temperature parameters (mean, variance, minimum, and maximum values) were estimated by a nested ANOVA of depth within shading.

TABLE 2. Effect of depth and shading treatments on temperature detected by an ANOVA of the records taken at common times for all field nests of *Podocnemis expansa*.

A) General effect

Temperature parameter	Treatment	SS	*F*	*P* <
Raw temperature	shading	9729.10	8845.10	0.00001
	depth	266.50	121.20	0.0001
Mean temperature	shading	15.64	25.90	0.0001
	depth	0.42	0.35	0.71
Standard deviation	shading	0.56	20.02	0.0004
	depth	0.67	11.96	0.0007
Minimum value	shading	4.62	11.41	0.004
	depth	0.32	0.40	0.68
Maximum value	shading	32.80	29.50	0.0001
	depth	7.54	3.39	0.06

B) Parameter estimates

Temperature parameter	Depth effect within:	β	*t*	*P*
Raw temperature	sun	−0.017	−15.55	0.0001
	shade	−0.0008	−0.68	0.49
Mean temperature	sun	−0.017	−0.84	0.41
	shade	−0.0008	−0.04	0.97
Standard deviation	sun	−0.021	−4.83	0.0002
	shade	−0.003	−0.75	0.46
Minimum value	sun	0.0133	0.82	0.42
	shade	0.0062	0.34	0.73
Maximum value	sun	−0.07	−2.57	0.02
	shade	−0.013	−0.43	0.67

RESULTS

Effect of experimental design on temperature

In general, temperature was higher in the sunny nests when compared to shaded nests, and temperature was lower and fluctuated less in deeper nests than in superficial nests. There was a significant interaction of shading and depth on nest temperature. A nested ANOVA of the raw hourly temperature records by depth within each shading treatment revealed a significant negative effect of depth on temperature for the sunny nests ($P < 0.0001$), and a negative but nonsignificant effect for the shaded nests. The same result was true for maximum temperature and for temperature variance. Mean and minimum temperatures were not affected by depth but were significantly higher in the sunny nests ($P < 0.0001$). This ANOVA was performed using the temperature records taken at common times for all field nests, natural and experimental (Table 2).

Climate changed during the nesting season such that nests in the same shading by depth treatment experienced different incubation temperatures if their oviposition day differed. For example, nests 3, 12, 14, and 16 were set on 3, 5, 25, and 29 November, respectively;

TABLE 3. Developmental stage according to Yntema (1968) of the *Podocnemis expansa* embryos sampled weekly from each field nest and constant temperature experiment.

Incubation conditions	Nest ID	Incubation week														Incubation time (d)†	Developmental rate (incubation time)$^{-1}$
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Laboratory temperature																	
Constant 30.5°C	···	11	14	17	20	···	24	25								58.2	0.45
Constant 32.5°C	···	11	13	18	21	23	25	···								49.4	0.53
Constant 33.5°C	···	12	14	19	21	24	25	···								48.5	0.54
Constant 34.5°C	···	12	15	18	21	23	25	···								46.7	0.56
Field treatment																	
Natural nests	8	12	14	17	18	21	23	25	26							58	0.45
Natural nests	15	11	13	17	20	22	24	25	26							63	0.42
Natural nests	18	11	13	17	20	22	···	25	26							59	0.44
Sun 15 cm	9	···	14	17	19	21	23	···	···	···	···					65	0.4
Sun 15 cm	17	11	13	17	19	21	23	···	···	···	···					63	0.41
Sun 15 cm	19	10	14	17	20	22	24	25	25	26	···					65	0.4
Sun 15 cm	22	···	14	16.5	19	21	23	24	25	···	26					70	0.37
Sun 30 cm	4	11	15	···	21	24	25	···	25	26						60	0.43
Sun 30 cm	7	11	12	17	···	21	23	25	25	26						64	0.41
Sun 45 cm	5	10	14	17.5	20	22	···	25	26	···						61	0.43
Sun 45 cm	10	10	13	17	20	22	23	24	25	26						65	0.4
Shade 15 cm	3	···	12	14	16	18	19	20	21	23	···	25	25	···	26	93	0.28
Shade 15 cm	12	···	11	14.5	16	17	19	19	21	23	23	25	25	26	···	94	0.28
Shade 15 cm	14	9	11	12	14	15	18	19	19	20	···	···	···	···	···		0.32
Shade 15 cm	16	11	12	14	14.5	16	17	18	20	23	25	···	···	···	···		0.36
Shade 30 cm	2	9	11	13	15	15	18	19	19	20	22	23	24	···			0.29
Shade 30 cm	6	···	12	15	17	18	20	21	21	23	24	25	25	26		93	0.28
Shade 30 cm	13	9	12	14	15	15	17	19	20	21	24	···	···	···			0.34
Shade 45 cm	11	10	11	14	15	17	18	20	21	23	24	···	25	···			0.3
Shade 45 cm	21	9	11	14	15	16	···	18	19	21	23	25	···	···			0.32

Notes: Stage 26 is the stage at hatching. Note the variation in development within each field treatment, which reflects variation in incubation temperatures due in part to differences in oviposition date. In general, constant temperatures yielded the fastest developmental trajectories with stage 25 attained by week 6 or 7. Natural nests were second, followed by the experimental nests in the sun, and shaded nests showed the slowest development of all treatments.

† For nests where all the embryos died, the cell for incubation time is blank.

all in the shade experiments at a depth of 15 cm. The first two nests experienced much higher temperatures during their first three weeks of incubation such that by stage 17 of development the four nests had accumulated strikingly different numbers of cumulative temperature units (342, 242, 0.3, and zero CTUs, respectively).

Effect of temperature on development

Embryos incubated at constant temperatures attained more advanced stages of development every week than any of the field treatments, with the exception of natural nests 8, 15, and 18 whose embryos developed at a similar rate (Table 3). Likewise, embryos from sunny nests developed faster than embryos in shaded nests (Table 3). Developmental rate (incubation time^{-1}) was positively associated with total mean temperature and total CTUs (Fig. 2, Table 4). A significantly better explanation of the variation in developmental rates, assessed by a test of the increment in the coefficient of determination (Sokal and Rohlf 1995), was obtained when using total CTUs plus the time spent at temperatures below 28°C prior to the thermosensitive period (TSP) in a multiple regression analysis ($R^2 = 0.88$; $F = 60$, $P \ll 0.001$; Table 4).

Incubation time increased in the shift-once experiments from 30.5°C to 34.5°C as the time of transfer was progressively delayed and the mean temperature decreased (Fig. 3). Incubation time also increased in the shift-twice experiments (34.5°-30.5°-34.5°C) as the time at the intermediate temperature was prolonged for each group of eggs and the mean temperature decreased (Fig. 3). The opposite trend was observed in the respective reciprocal experiments (Fig. 3).

Depth and shade had a drastic effect on embryonic development. Development was slowed more by shading than by depth (Table 3). All field nests (including natural nests) exhibited some degree of mortality, which increased with shading (Tables 1 and 4). In fact, only three shaded nests produced live hatchlings (nest numbered 3, 6, and 12). These three nests were nests that experienced temperatures above 28°C, particularly during the first three weeks of incubation. All other shaded nests, which experienced temperatures mostly below 28°C, failed to hatch. This result indicated that temperatures experienced at early stages had a crucial

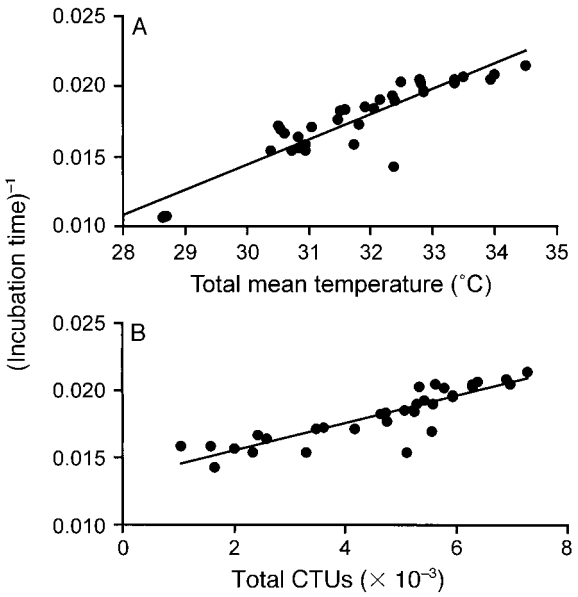

FIG. 2. Developmental rate of *Podocnemis expansa* embryos as a function of (A) mean temperature and (B) thousands of CTUs during the entire incubation period.

effect on development. Developmental rate prior to the TSP in the field nests was affected both by the CTUs (positive effect) and by the number of hours below 28°C (negative effect), as suggested by the significant multiple regression (Table 4). Additionally, mortality in the field nests increased with the amount of time (number of hours) spent below 28°C from oviposition to stage 17, as indicated by the positive logistic regression (Table 4). Embryos that died in the shaded experiments died at earlier developmental stages than those that died in sunny conditions (Table 5). The CTUs per embryological stage were highest at constant incubation conditions, followed by the shift experiments, the field experiments in the sun or natural nests, and were the lowest in the shading experiments (Fig. 4).

Temperature and sex ratios

The proportion of females produced increased with increasing constant temperature in the laboratory (Fig. 5). Sex ratios produced at 30.5°C and 32.5°C seemed more female biased during this study than in 1991 (Fig. 5; Lance et al. 1994). The pivotal temperature calculated by maximum likelihood and by inverse prediction from logistic regression, was 32.6°C using raw data from 1991 (N. Valenzuela, *unpublished data*) and from this study. The lower and upper 95% confidence limits of the pivotal temperature were 32.2°C and 33.2°C, respectively. Using data from the present study alone, the critical temperature was 32.2°C by both methods and the 95% confidence limits were 31.4°C and 32.9°C.

The sex ratios produced in some shift experiments were concordant with the expected pattern, while sex ratios produced in other shift experiments were not. Namely, a clear increase in female production was observed in the shift-once experiment from 34.5°C to 30.5°C (Fig. 1) as the transfer time was progressively delayed from 14 to 42 d. This pattern was expected because the continuous delay in transfer time induced the steady increase in mean temperature and exposed

TABLE 4. Results of the regression analyses performed in this study of *Podocnemis expansa*.

Response variable†	Predictor variable‡	Analysis	*n*	Regression coefficient	R^2	*P*
$(\text{Time to hatching})^{-1}$	mean temperature	regression	33	0.002	0.85	0.0001
$(\text{Time to hatching})^{-1}$	total CTU ($\times 10^{-3}$)	regression	33	0.0013	0.64	0.0001
$(\text{Time to hatching})^{-1}$	total CTU ($\times 10^{-3}$) and hours $<28°C_{0-17}$	multiple regression	33	0.001, -1.2×10^{-5}	0.88	0.0001
Mortality field	hours $<28°C_{0-17}$	logistic regression	42	0.0067	0.36	0.00001
$(\text{Days to reach stage 17})^{-1}$	CTU_{0-17} ($\times 10^{-3}$) and hours $<28°C_{0-17}$	multiple regression	42	0.007, −0.00002	0.94	0.00001
Sex ratio (% females) laboratory	CTU_{17-24} ($\times 10^{-3}$)	logistic regression	22	0.64	0.07	0.0002
Sex ratio laboratory and field	CTU_{17-24} ($\times 10^{-3}$)	logistic regression	33	0.44	0.03	0.0001
Sex ratio laboratory and field	CTU_{17-24} ($\times 10^{-3}$) and hours $<28°C_{0-17}$	multiple logistic regression	33	0.5, −0.013	0.05	0.0006
Developmental stage	CTU ($\times 10^{-3}$)	regression	112	1.7	0.67	0.00001
Sex ratio laboratory and field	$(CTU_{17-24})^{1.3}$ ($\times 10^{-3}$)	logistic regression	33	0.58	0.05	0.0001
Sex ratio laboratory and field	$(CTU_{17-24})^{1.3}$ ($\times 10^{-3}$) and hours. $<28°C_{0-17}$	multiple logistic regression	33	0.59, −0.01	0.06	0.001
Clutch size	egg-chamber depth	regression	84	1.67	0.19	0.0001

† $(\text{Time to hatching})^{-1}$ = developmental rate for the entire incubation period; $(\text{Days to reach stage 17})^{-1}$ = developmental rate from oviposition to stage 17; Sex ratio laboratory and field = percentage females for the laboratory and field combined data set.

‡ Total CTU ($\times 10^{-3}$) = CTUs calculated from oviposition to hatching divided by 1000; CTU_{17-24} ($\times 10^{-3}$) = CTUs calculated for the thermosensitive period (between stages 17 and 24 of development divided by 1000; hours $<28°C_{0-17}$ = time spent at temperatures below 28°C prior to the thermosensitive period (from oviposition to stage 17).

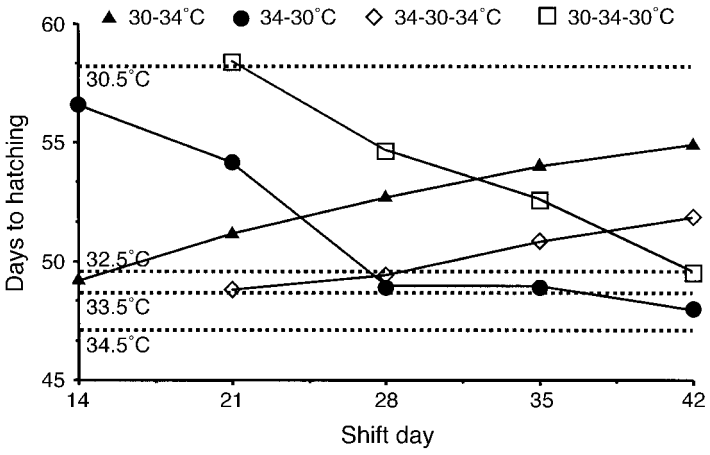

FIG. 3. Changes in incubation time of *Podocnemis expansa* with shift-temperature conditions. Shift-once experiments are represented by solid symbols, and shift-twice experiments by open symbols. Each data point represents a group of eggs subjected to a shift experiment with the transfer time indicated in the horizontal axis. Incubation times at constant temperatures are depicted by dotted lines for comparison purposes.

each group of embryos to greater periods of female-producing temperatures (Fig. 1). Likewise, although not as apparent, the proportion of females decreased in the shift-twice experiment 34.5°-30.5°-34.5°C as the second transfer time was delayed (Fig. 1). A decreasing pattern was expected in this instance because each group of embryos was exposed to shorter periods of female-inducing temperatures at the same time that the mean temperature was reduced for each sequential transfer group. In contrast, the reciprocal of these two experiments (shift-once from 30.5°C to 34.5°C, and the shift-twice 30.5°-34.5°-30.5°C) showed the expected decrease and increase in the percentage of females only slightly (Fig. 1).

Sex ratio data combined with the information on embryonic development over time in these shift experiments were used to roughly estimate the thermosensitive period (TSP), which is tentatively comprised of stages 17–24 of development. CTUs were then calculated for this period of time ($CTU_{17–24}$) for all artificial incubation experiments including constant temperature, shift-once, and shift-twice treatments. This temperature variable significantly explained the sex ratios produced in the laboratory as indicated by the logistic regression analysis (Table 4).

When $CTU_{17–24}$ values from the field nests were included in the logistic regression analysis, a positive significant relationship was detected between this tem-

TABLE 5. Number of *Podocnemis expansa* embryos that died at different developmental stages during incubation in the field.

Treatment	Nest ID	Undeveloped eggs†	Developmental stage‡								Eggs eaten§	Hatchlings	
			<10	14	15	19	20	22	25	26		Dead‖	Alive
Sun 15 cm	9	12	1	…	…	…	2	1	6	10	14	10	35
Sun 15 cm	22	11	…	…	…	8	10	…	…	…	…	1	53
Sun 15 cm	19	9	…	…	…	1	…	…	…	…	3	11	53
Sun 15 cm	17	10	…	…	1	1	…	…	…	1	4	8	62
Sun 30 cm	4	37	4	…	…	…	1	…	6	5	3	7	31
Sun 30 cm	7	14	…	…	…	3	…	2	1	…	…	…	62
Sun 45 cm	5	26	…	…	…	1	…	…	1	1	27	1	56
Sun 45 cm	10	35	…	…	…	…	…	1	5	2	3	1	42
Natural	8	2	…	…	…	…	…	…	…	1	35	7	33
Natural	18	2	…	1	…	…	…	…	…	2	…	1	91
Natural	15	7	…	…	…	…	…	…	…	3	2	6	60
Shade 15 cm	3	8	2	…	…	1	1	3	7	33	12	13	7
Shade 15 cm	12	6	1	1	…	2	12	11	20	15	9	…	1
Shade 15 cm	14	24	1	1	1	28	10	1	…	…	…	…	…
Shade 15 cm	16	24	6	3	14	32	7	1	…	…	8	…	…
Shade 30 cm	2	27		1	7	20	11	5	4	…	8	…	…
Shade 30 cm	6	7	1		2	2	2	6	16	24	1	4	20
Shade 30 cm	13	27	3	3	9	24	19	5	1	…	1	…	…
Shade 45 cm	11	40	3	1	1	1	4	7	4	7	10	…	…
Shade 45 cm	21	42	2	1	2	6	5	3	6	…	20	…	…

† Undeveloped eggs are those for which no embryonic development was detected.
‡ Developmental stages are classified according to Yntema (1968).
§ Eggs eaten are those that were either attacked by burrowing crickets or showed advanced fungal growth.
‖ Dead hatchlings correspond to individuals found dead within the egg chamber after hatching but before emergence.

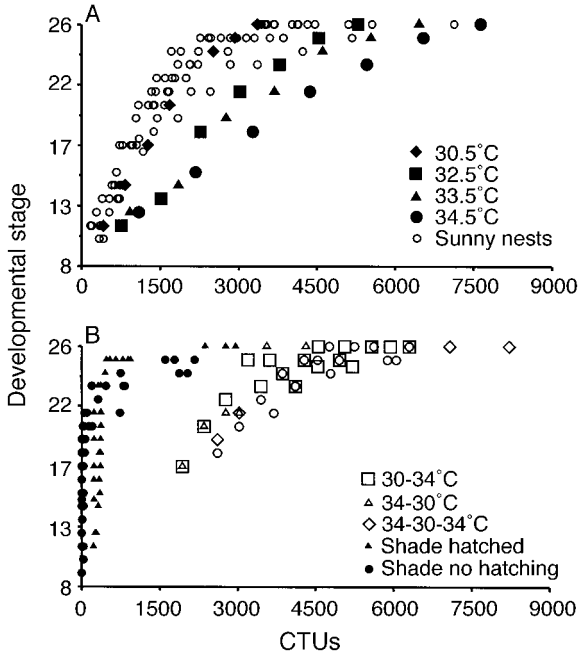

FIG. 4. Developmental stage of *Podocnemis expansa* embryos as a function of CTUs (untransformed values) for each laboratory and field treatment. (A) Data from constant temperature treatments (solid symbols) and field nests in the sun (open symbols). (B) Data from shift-once and shift-twice experiments (open symbols) and field shaded nests that did and did not produce hatchlings (solid symbols).

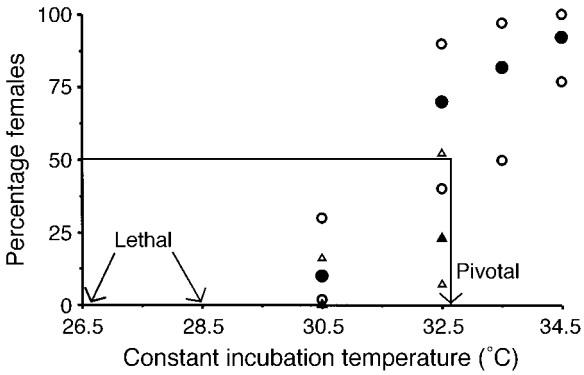

FIG. 5. Percentage female *Podocnemis expansa* produced under constant incubation temperatures in the laboratory. Solid triangles symbolize data from 1991 including lethal temperatures (from Lance et al. 1994), and solid circles represent data from this study. The pivotal temperature was calculated for the combined data set. Open symbols indicate the upper and lower limits of the 95% confidence interval from a binomial distribution for the sex ratios estimated at each temperature in 1991 (triangles) and in this study (circles).

perature variable and sex ratios (Table 4). It should be noted that the coefficients of determination were quite small, indicating that a significant proportion of the variation in sex ratio was left unexplained. The analysis of covariance indicated that there was a significant interaction of nest origin (field or laboratory) and CTUs on sex ratio ($P < 0.002$). The nested analysis of CTUs and sex ratios within laboratory was positive and significant ($P < 0.0003$), while the relationship for just the field nests was not significant and negative ($P < 0.43$); but most field data points were within the range of variation described by the laboratory data alone (Fig. 6). Some field nests had to be excluded from this analysis. Nest number 12 only produced one living hatchling, and nests 3 and 6 were outliers. These three nests were the only shaded nests that produced live hatchlings. However, nests 3 and 6 were the coldest of all field nests in which hatching occurred, yet they produced the most females (83.3% and 73.3%) according to a TSDII pattern, and thus had to be removed from the logistic regression analysis. Table 1 summarizes the sex ratios obtained in all experiments and natural nests during this study.

Finally, the time below 28°C prior to the TSP was incorporated into the model (Table 4) and it explained more residual variation than the time below 28°C just during the TSP or during stages 0–24 of development. However, the multiple regression for just the field data was still not significant, and the trend of sex ratio vs. CTUs was still negative. Soil water content remained quite uniform during the entire incubation. Readings ranged between 95% and 100% water content, regardless of the time of day or whether records were taken before or after rain. Not surprisingly, soil water content did not explain additional variation in field sex ratios. The implicit assumption of the logistic regression model that there is a linear relationship between CTUs and development was met by the data. Only the linear component of the polynomial regression analysis of developmental stages on CTUs was significant (Table 4).

In order to test the sensitivity of the model, I explored some alternatives to the original CTUs defined in the experimental design using the laboratory data alone. First, several cutoffs above the survival threshold and up to the pivotal temperature were used instead of 28°C (28.5°, 29°, 29.5°, 30°, 30.5°, 31°, 31.5°, 32°, and 32.5°C). The best fit was obtained using 31°C, but

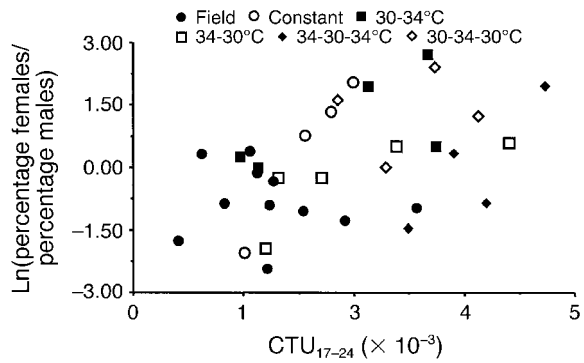

FIG. 6. Field and laboratory *Podocnemis expansa* sex ratio logits as a function of thousands of CTUs during the thermosensitive period (embryonic stages 17–24).

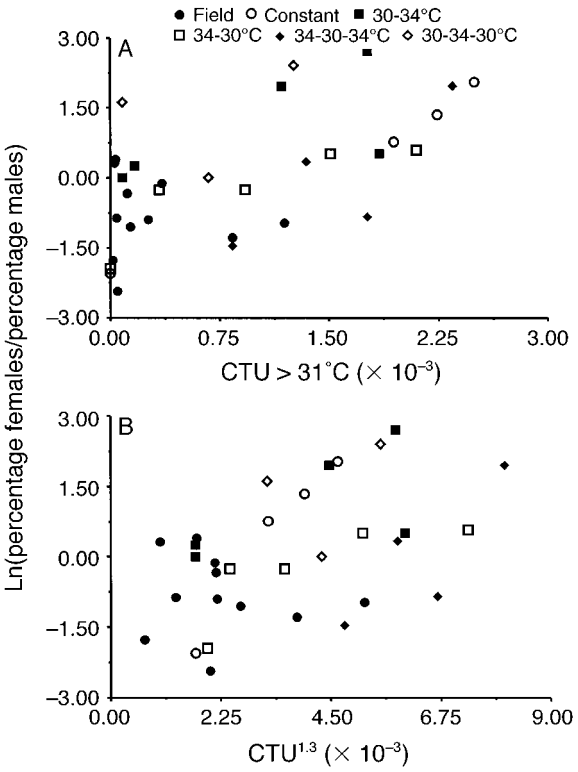

FIG. 7. (A) Relationship between *Podocnemis expansa* sex ratio and thousands of CTUs, calculated using the alternative cutoffs to 31°C. (B) Exponential relationship between sex ratio and $CTU^{1.3}$ ($\times 10^{-3}$). CTUs in (A) and (B) correspond to the thermosensitive period.

this variable did not explain sex ratio for the field data alone (Fig. 7). With field and laboratory data combined, the fit increased gradually from 28°C to 32°C; but this happened because the field data were increasingly removed from the laboratory data, causing a better total fit, but leaving more of the field variation unexplained (Fig. 7). The alternative that no threshold may give a better fit was explored for the laboratory as well, but the sum of temperatures between stages 17 and 24 reduced the fit of the model. Finally, I explored the possible nonlinear relationship between CTUs and sex ratio by raising CTUs to an arbitrary power of 0.7 or 1.3 when calculating $CTU_{17–24}$, to test whether the relationship was decelerated or accelerated with respect to temperature. The fit of the model was reduced with the power of 0.7 but increased when using the power of 1.3 for the laboratory data alone and also for the laboratory and field data combined (Table 4, Fig. 7). This suggests that the effect of higher temperatures in the production of females is disproportionately greater. The multiple logistic regression of sex ratios on the combination of CTU between stages 17 and 24 $(CTU_{17–24})^{1.3}$ and the time below 28°C prior to the TSP gave the best fit for the combined data set (Table 4).

DISCUSSION

One of the more intriguing but poorly understood sex-determining mechanisms is temperature-dependent sex determination (TSD), which occurs primarily in reptiles (reviewed in Bull 1980, Ewert and Nelson 1991, Janzen and Paukstis 1991, Ewert et al. 1994, Lang and Andrews 1994, Viets et al. 1994, Shine 1999). TSD is one environmental sex-determining mechanism that can potentially produce skewed sex ratios, in direct contrast with Fisherian expectations of 1:1 sex ratios (Fisher 1930), and with significant potential effects on population parameters. Despite intensive research on this life history trait over the last two decades, the relationship between fluctuating temperatures and sex determination in nature remains obscure (e.g., Georges 1989, Georges et al. 1994, Valenzuela et al. 1997). The present study attempted to provide a better understanding of TSD in nature by performing a series of simultaneous field and laboratory experiments using constant, shift, and fluctuating temperature regimes, and monitoring temperature, embryological development, and sex ratio concurrently. I presented a model of sex determination where sex ratio (% females) is an increasing function of the cumulative exposure to temperatures above the survival threshold during the temperature sensitive period $(CTU_{17–24})^{1.3}$, and a decreasing function of the time spent at temperatures below the survival threshold prior to the temperature sensitive period (hours $<28°C_{0–17}$). Because the R^2 values reported here were low, the model should be considered tentative, and its applicability should be confirmed by further field and laboratory studies.

One advantage of using cumulative temperature units (CTUs) to explain sex ratios rather than using the mean temperature, is that it becomes possible to predict the onset of the thermosensitive period (TSP) from the trajectory of CTUs over time (Fig. 8), while with the mean temperature it is necessary to know this period of time beforehand. The end of the TSP (stage 24) can also be predicted from the daily accretion of CTUs but with less accuracy than for the onset (Fig. 8). The CTUs used here are a modification of the heat-unit accumulation and the derived degree-days concept, which have been used in the past to explain biological processes (Arnold 1960, Baskerville and Emin 1969, Schwarzkopf and Brooks 1985). Degree-days are calculated as the number of days the mean temperature is above a given threshold multiplied by the mean temperature during those same days. Unlike degree-days, the CTUs used in this study make no assumptions about the shape of the temperature profiles, because the records were taken every hour and thus allowed the calculation of the area directly from the actual profiles themselves.

I observed significant differences and similarities in the effects of artificial incubation regimes compared to naturally fluctuating temperatures. The lower survival threshold was similar under artificial and natural in-

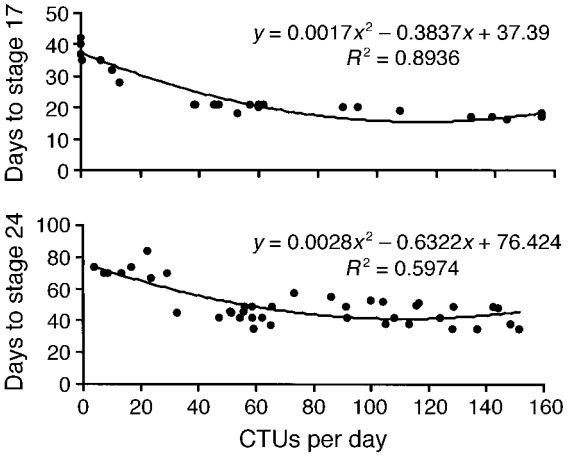

FIG. 8. Chronological onset (embryonic stage 17) and end (embryonic stage 24) of the thermosensitive period of *Podocnemis expansa* in the field and the laboratory as a function of the rate of accretion of CTUs (untransformed values) for that period of time (oviposition to stage 17 or to stage 24).

cubation conditions. Striking effects of nest depth on thermal conditions were noted that are ecologically meaningful for natural populations. Pulses of extreme temperatures were not lethal under fluctuating regimes in nature but affected developmental rates and sex determination. The failure of previous models to explain sex determination in nature for *Podocnemis expansa* is apparently due to the oversimplification of laboratory experiments that overlook crucial complex multivariate interactions of naturally fluctuating temperatures, development, and sex differentiation.

Effects of depth and shading treatments

As expected, temperatures were colder in the shade than in the sun and this was reflected in a lower mean and minimum temperature in shaded nests. Natural nests of this species are not typically located in heavily vegetated areas (N. Valenzuela, *personal observation*) where they could be shaded most of the time. The majority of nests are laid in open parts of the beach. Thus shaded nests do not resemble typical natural incubation conditions for this species, which is not surprising given the extreme mortality rates caused by this treatment. In contrast, some turtle species commonly nest in vegetated areas; e.g., *Chelonia mydas* (Bjorndal and Bolten 1992), and *Chrysemys picta* (Janzen 1994). In both cases the resulting shading reduced nest temperatures, causing male biases without seemingly increasing mortality as observed for *P. expansa.*

Depth in the sunny experiments significantly lowered nest temperatures. In general, the deeper the nest the colder and less variable the incubation conditions. Average nest depth of natural nests in this population is 46.2 cm (21–71 cm; $n = 188$) (von Hildebrand et al. 1997, Valenzuela 2001). Therefore, experimental nests had depths (35, 50, and 65 cm) comparable to natural ones. The relationship between depth and sex ratio for the sunny nests exhibited a somewhat concave pattern (Table 1), reflecting the effect of additional factors in this relationship. One of those factors may be seasonal climatic changes given that experimental nests were purposely set at different dates in order to break developmental synchrony, and thus the sex ratios produced were probably the partial result of the interaction between depth and onset timing. This observation also reflects the importance that seasonal or annual climatic changes can have on nest temperature by overriding the effect that nest site choice has at the microgeographic scale since nest depth is positively correlated with female size in this species (Valenzuela 2001).

Survival threshold

The observation that no hatching occurred in shaded field nests that experienced incubation temperatures almost exclusively below 28°C confirms that 28°C represents the survival threshold for this species as postulated in Valenzuela et al. (1997). This finding also validates a posteriori the use of the laboratory-based survival threshold in the definition of CTUs in the experimental design. Temperatures early in the incubation period had a crucial role in altering and thus determining the fate of embryonic development. Hatching in the shaded experiments occurred only in those nests whose temperature records exceeded 28°C during the first three weeks of incubation, even when temperatures fell below 28°C later in development.

Temperature and developmental rate

Development was faster at higher constant incubation temperatures in the laboratory, and at higher mean temperatures for the field and laboratory combined. This result was consistent with data from this (Valenzuela et al. 1997) and other turtle species (e.g., Birchard and Reiber 1996, Booth 1998). Developmental rate was retarded in the field as compared to laboratory experiments. This effect was not caused by absolute higher incubation temperatures in the laboratory, but rather by the amount of time field nests experienced temperatures above and below the survival threshold. In fact, temperature in some field nests reached 40°C, yet they exhibited lower overall developmental rates than laboratory experiments in which the maximum temperature was only 34.5°C. On the other hand, the number of hours below the survival threshold explained additional variation in developmental rates when added to the CTUs in the multiple regression. It seems clear that embryos can survive temperatures under fluctuating conditions that would be lethal if constant. Temperatures well below or above the lower and upper survival thresholds are common in natural nests, but their lethal effects seemed dependent on duration of exposure and not just magnitude. Their influence was apparently compensated by the exposure to temperatures within the optimal range for development, such

that daily pulses of low (and possibly very high) temperatures in nature were not lethal but only retarded development.

Temperature and sex determination

The resulting pivotal temperature, 32.6°C, is the highest of any TSD turtle species studied to date, yet it is lower than the pivotal temperature for *Crocodylus porosus* (33°–34°C; Mohanty-Hejmadi et al. 1999). The pivotal temperature of *Podocnemis unifilis*, a congeneric species that nests frequently in the same beaches during *P. expansa*'s nesting season, has a value between 31°C and 32°C (Souza and Vogt 1994). The next highest pivotal temperature calculated for a turtle species, 31.8°C (Spotila et al. 1994), corresponds to the desert tortoise, *Gopherus agassizii*. Pivotal temperatures of all sea turtle species are close to 29°C (Davenport 1997) although a recent study reports 31°C for *Lepidochelys olivacea* (Wibbels et al. 1998). Pivotal temperatures for other species range between 25°C and 30°C (Ewert and Nelson 1991, Ewert et al. 1994, Girondot 1999). Since 30.5°C produced 4.5% females (after combining data from 1991 and 1997), and 28.5°C was lethal (Valenzuela et al. 1997), temperatures producing 100% males in *P. expansa* seem to have a very restricted range around 29.5°C.

The model of sex determination presented here (percent female is an increasing function of $(CTU_{17–24})^{1.3}$ and a decreasing function of the hours $<28°C_{0–17}$) successfully explained sex ratio production in the laboratory, in the laboratory and field combined, but not in the field alone. Furthermore, the relationship between CTUs accumulated during stages 17–24 and sex ratio for field data showed a negative (although not significant) trend. Although most field data points were within the range of variation described solely by the laboratory data, they concentrated in the lower half of the distribution of sex ratios. Cold climatic conditions during the present study may be responsible for the low proportion of females produced in the field, 6–60% females compared to 73–100% females produced in the study region during the 1993–1994 reproductive season (Valenzuela et al. 1997), and seemingly 100% females produced in 1991 (Lance et al. 1994). As mentioned before (see *Temperature and developmental rate*), developmental rate was slowed down by the amount of time that temperatures fell below the survival threshold. Interestingly, the time spent at temperatures below 28°C prior to the TSP was the variable that best explained the residual variation of all sex ratio data combined, as would be expected if sex determination in natural nests is related to developmental rate (Georges 1989). However, the relationship for just the field was not significant, probably due in part to low sample size. Interestingly, developmental rate per se did not explain additional variation in sex ratio.

It seems that temperature has two ways of affecting sex ratios. First, the cumulative exposure to temperatures above 28°C during the TSP has a positive effect on the proportion of females produced. Second, time spent below the survival threshold has a negative influence on developmental rate and proportion of females, an influence that is exerted mainly at the beginning of the incubation period in the field. This is consistent with previous studies that demonstrated that the effects of temperature on growth and metabolism in reptiles are mainly restricted to early development (Birchard and Reiber 1996, and references therein).

Additional factors in sex determination

Even though a significant effect was detected in the combined data set, the relatively low R^2 values (Table 4) for the relationship between sex ratio and CTUs indicate that other factors are likely to be involved in sex determination. The alternative cutoffs to 28°C explored in the sensitivity analysis (28.5°–32.5°C) improved the fit of the model by increasingly pulling the field data away from the laboratory data, leaving more of the field variation unexplained. Therefore, this improvement was a statistical artifact and not a better biological explanation of the data. The second alternative explored showed that there was an accelerated relationship between CTUs and sex ratios (Table 4), such that higher temperatures had a disproportionately larger effect on the production of females. Such differential effect of temperature has been described previously for other TSD species (Yntema 1979, Johnston et al. 1995).

High mortality rates were observed across all field and laboratory experiments (Table 1) and could have strong effects on sex ratios if mortality was biased toward one sex or the other. Much lower mortality rates (5%) were recorded in 1993 (Valenzuela et al. 1997) for experimental and natural nests that were comparable to the sunny experiments and control nests reported here. One possible explanation is that the experiments conducted in the present study required extra handling compared to those reported in Valenzuela et al. (1997) and this increased the general mortality. However, natural mortality rates range between 1% and 63% (von Hildebrand et al. 1997) and mortality in completely undisturbed natural nests during my study was 24% (range = 0–100%, $n = 41$), suggesting that 1997 may simply have been a year of higher natural mortality. The high mortality rates during laboratory experiments may be explained in part as the result of the hardship imposed on the eggs during transportation and handling. I could not, however, determine to what extent the mortality was biased toward one or the other sex since dead embryos were not preserved, but it should be noted that not all the embryos that died had reached the TSP. Mortality rates of embryos between stage 17 and hatching were much lower than total mortality (Table 1) and constitute the relevant values regarding potential sex-biased mortality.

Another possible explanation for the discrepancy in

trends between laboratory and field could be the high mortality in the shaded experiments, which reduced the sample size in half. Field experimental nests were subjected to treatments designed to increase variation in temperature conditions, and consequently to produce sex ratios ranging from mostly males to mostly females. These treatments resulted in a wide variation of incubation conditions but the sex ratios produced in the field were nonetheless restricted to 13–58% females. The natural nests monitored produced between 6% and 60% females. This may explain the interaction found between CTUs and nest origin (field or laboratory) on sex ratios. On one hand, pooling both data sets (field and laboratory) could be justified since the model was conceived a priori, and because sex determination should occur through the same mechanism in both settings. On the other hand, the data suggest that sex determination in the field is a more complex process than in the laboratory, such that models derived from laboratory data may be inappropriate for field data. Further studies with larger sample sizes and additional species are required to make any conclusive statement.

There is also the possibility that the timing of the TSP could be radically different in the field due to the nature of the temperature fluctuations or to some other undetermined factor. The TSP was determined from the shift experiments as stages 17–24 of development, using the following criteria. From constant incubation temperature results, 30.5°C was expected to produce ~10% females, whereas 34.5°C was expected to produce ~90% females. In the shift-once experiments, a sex ratio equal to that expected by the second temperature indicated that the TSP had not started by the transfer time, whereas a sex ratio equal to that expected by the first temperature indicated that the TSP had ended by the transfer time. Sex ratios intermediate to those expected by either temperature indicated that the TSP was active at the transfer time. Sample sizes from these experiments were small (Fig. 1) and the results, therefore, were suggestive but not conclusive. In the shift from 30.5°C to 34.5°C, changes were evidenced after stage 17. Only three hatchlings were available from the third group shifted and it was possible that by that time (stage 20) the thermosensitive period had already started. In the shift from 34.5°C to 30.5°C, the TSP seemed to have started sometime between stages 15 and 18. The TSP may have been still active by stage 24 in both cases, but stage 24 is a very advanced developmental stage and the TSP could have ended by then. If the TSP is truly comprised of embryonic stages 17–24 in the laboratory and the field, then the TSP in the laboratory started in the second third of incubation and lasted for 50% of the incubation time on average, while in the field it started in the second third and lasted for 40% of the incubation time. Because sample sizes from the shift experiments were extremely low, the TSP estimate is tentative. The TSP in turtles has been found to occur in the middle third of the incubation period (Bull 1983, Janzen and Paukstis 1991), but it was suggested that TSP in the field can be longer than under laboratory conditions (Souza and Vogt 1994).

Within-nest variation in temperatures may also add to the variation in sex ratios. The proportion of females tended to decrease with increasing depth, although this trend was not statistically significant. The experimental design of nest depths was such that eggs in the bottom of the egg chamber of a given treatment were at the same depth as the upper eggs in the next deepest treatment. A significant effect of nest depth on temperature was detected with the treatments used, such that within a single egg chamber there may be differences in incubation temperatures between the upper and bottom eggs. This within-nest variation could not be accounted for with the number of nests available for this study, and it most likely adds to the unexplained variation in sex ratio (Georges 1989). This type of within-nest depth effect on nest temperature and ultimately sex ratios has been reported for the turtles *Chelonia mydas* (Kaska et al. 1998), *Caretta caretta* (Hanson et al. 1998, Kaska et al. 1998), *Carettochelys insculpta* (Georges 1992), and *Chelydra serpentina* (Wilhoft et al. 1983).

Temperature-dependent sex determination (TSD) mode

Contrary to expectation, the two nests that experienced the coldest temperatures in the field (numbered 3 and 6) produced the most females. This striking finding suggests that *P. expansa* may be a TSDII species. TSDII is also present in two African-Malagasy species of the same family Pelomedusidae (*Pelomedusa subrufra* and *Pelusios castaneus*) (Ewert and Nelson 1991), while *Podocnemis unifilis* has TSDIa (Souza and Vogt 1994). The expression of TSDII in the field in *P. expansa* seems truncated by the general lethality of low temperatures, such that it is expressed as TSDIa in nature. Similar observations were made for *P. erythrocephala* (R. C. Vogt, *unpublished data*). These observations are consistent with the hypothesis that TSDII is the ancestral condition from which TSDIa and TSDIb evolved, by the species shifting their sensitivity to different portions of the temperature range and by some extreme temperature becoming lethal. In some cases, TSDII may be an experimental artifact. For example, some species reported as TSDII from laboratory experiments have been incubated at lower temperatures only during the TSP, because exposure to those temperatures for an extended period of time is lethal (Ewert and Nelson 1991, Ewert et al. 1994). *Podocnemis expansa* and *P. erythrocephala* could be two such species. Some other species may rarely encounter low enough temperatures for TSDII to be expressed in nature (e.g., *Kinosternon leucostomum*; Ewert et al. 1994). Therefore, these purportedly TSDII species in which the production of low-temperature females is precluded would be functionally TSDIa. These observations raise ques-

tions about the relevance of the current classification system, and renew the challenge of understanding the ecological significance and evolutionary potential of temperature-dependent sex determination.

Acknowledgments

I specially thank biologists E. Rodríguez and L. K. Agudelo for their invaluable help during the data collection in the field and the laboratory, respectively. I am grateful to P. von Hildebrand for his support, and to D. Guzmán, D. Muñoz, and A. Cortés for their unconditional logistical help. I thank the Nonuya people from Peña Roja in the Caquetá River of Colombia for allowing our stay, and for participating in the fieldwork of this study. M. E. Patarroyo kindly provided the equipment and facilities to perform the radioimmunoassay. I also thank C. Janson, D. Dykhuizen, and D. C. Adams for their suggestions about data analysis, and F. J. Janzen, A. Bronikowski, and R. Ackerman for improvement of the manuscript. This work was funded in part by Colciencias COD 6218-13-143-95 RC-288-96 (Colombia), the National Science Foundation IBN-9800679, the Ford Foundation Grant 960-0929, and Fundación Puerto Rastrojo (Colombia), and is contribution 1081 from the Program in Ecology and Evolution at the State University of New York at Stony Brook.

Literature Cited

Arnold, C. Y. 1960. Maximum–minimum temperature as a basis for computing heat units. American Society of Horticultural Sciences **76**:682–692.

Baskerville, G. L., and P. Emin. 1969. Rapid estimation of heat accumulation from maximum and minimum temperatures. Ecology **50**:514–517.

Birchard, G. F., and C. L. Reiber. 1996. Heart rate during development in the turtle embryo: effect of temperature. Journal of Comparative Physiology B **166**:461–466.

Bjorndal, K. A., and A. B. Bolten. 1992. Spatial distribution of green turtle *Chelonia mydas* nests at Tortuguero, Costa Rica. Copeia 1992:45–53.

Boomsma, J. J. 1989. Sex-investment ratios in ants: has female bias been systematically overestimated? American Naturalist **133**:517–532.

Booth, D. T. 1998. Effects of incubation temperature on the energetics of embryonic development and hatchling morphology in the Brisbane river turtle *Emydura signata*. Journal of Comparative Physiology B **168**:399–404.

Bull, J. J. 1980. Sex determination in reptiles. The Quarterly Review of Biology **55**:3–21.

Bull, J. J. 1983. Evolution of sex determining mechanisms. Benjamin/Cummings, Menlo Park, California, USA.

Bull, J. J. 1985. Sex ratio and nest temperature in turtles comparing field and laboratory data. Ecology **66**:1115–1122.

Bulmer, M. 1994. Theoretical evolutionary ecology. Sinauer Associates, Sunderland, Massachusetts, USA.

Charnov, E. L. 1982. The theory of sex allocation. Princeton University Press, Princeton, New Jersey, USA.

Clark, A. B. 1978. Sex ratio and local resource competition in a prosimian primate. Science **201**:163–165.

Clutton-Brock, T. H., S. D. Albon, and F. E. Guinness. 1984. Maternal dominance, breeding success, and birth sex ratios in red deer. Nature **308**:358–360.

Conover, D. O. 1984. Adaptive significance of temperature-dependent sex determination in a fish. American Naturalist **123**:297–313.

Darwin, C. 1871. The descent of man and selection in relation to sex. John Murray, London, UK.

Davenport, J. 1997. Temperature and the life-history strategies of sea turtles. Journal of Thermal Biology **22**:479–488.

Ewert, M. A., D. R. Jackson, and C. E. Nelson. 1994. Patterns of temperature sex determination in turtles. The Journal of Experimental Zoology **270**:3–15.

Ewert, M. A., and C. E. Nelson. 1991. Sex determination in turtles: diverse patterns and possible adaptive values. Copeia 1991:50–69.

Fisher, R. A. 1930. The genetical theory of natural selection. Oxford University Press, Oxford, UK.

Georges, A. 1989. Female turtles from hot nests: is it duration of incubation or proportion of development at high temperatures that matters? Oecologia **81**:323–329.

Georges, A. 1992. Thermal characteristics and sex determination in field nests of the pig-nosed turtle, *Carettochelys insculpta* (Chelonia, Carettochelydidae), from Northern Australia. Australian Journal of Zoology **40**:511–521.

Georges, A., C. Limpus, and R. Stoutjesdijk. 1994. Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. Journal of Experimental Zoology **270**: 432–444.

Girondot, M. 1999. Statistical description of temperature-dependent sex determination using maximum likelihood. Evolutionary Ecology Research **1**:479–486.

Hamilton, W. D. 1967. Extraordinary sex ratios. Science **156**: 477–488.

Hanson, J., T. Wibbels, and R. E. Martin. 1998. Predicted female bias in sex ratios of hatchling loggerhead sea turtles from a Florida nesting beach. Canadian Journal of Zoology **76**:1850–1861.

Janzen, F. J. 1994. Vegetational cover predicts the sex ratio of hatchling turtles in natural nests. Ecology **75**:1593–1599.

Janzen, F. J., and G. L. Paukstis. 1991. Environmental sex determination in reptiles: ecology, evolution and experimental design. Quarterly Review of Biology **66**:149–179.

Johnson, C. N. 1988. Dispersal and the sex ratio at birth in primates. Nature **332**:726–728.

Johnston, C. M., M. Barnett, and P. T. Sharpe. 1995. The molecular biology of temperature-dependent sex determination. Philosophical Transactions of the Royal Society of London B **350**:297–304.

Karlin, S., and S. Lessard. 1986. Theoretical studies on sex ratio evolution. Princeton University Press, Princeton, New Jersey, USA.

Kaska, Y., R. Downie, R. Tippett, and R.W. Furness. 1998. Temperatures of green turtle (*Chelonia mydas*) and loggerhead turtle (*Caretta caretta*). Canadian Journal of Zoology **76**:723–729.

Lance, V. A., N. Valenzuela, and P. von Hildebrand. 1994. A hormonal method to determine sex of hatchling giant river turtles, *Podocnemis expansa*: application to endangered species. Journal of Experimental Zoology **270**:16A.

Lang, J. W., and H. V. Andrews. 1994. Temperature-dependent sex determination in Crocodilians. Journal of Experimental Zoology **270**:28–44.

Mohanty-Hejmadi, P., S. K. Dutta, D. Dey, D. P. Rath, R. L. Rath, and S. Kar. 1999. Temperature-dependent sex determination in the salt-water crocodile, *Crocodylus porosus* Schneider. Current Science **76**:695–696.

Mrosovsky, N., and C. Pieau. 1991. Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. Amphibia–Reptilia **12**:169–179.

Mrosovsky, N., and J. Provancha. 1989. Sex-ratio of loggerhead sea turtles hatching on a Florida beach. Canadian Journal of Zoology **67**:2533–2539.

Mrosovsky, N., and J. Provancha. 1992. Sex ratio of hatchling loggerhead sea turtles: data and estimates from a 5-year study. Canadian Journal of Zoology **70**:530–538.

Nonacs, P. 1986. Ant reproductive strategies and sex allocation theory. Quarterly Review of Biology **61**:1–12.

Pieau, C. 1982. Modalities of the action of temperature on

sexual differentiation in field-developing embryos of the European pond turtle *Emys orbicularis* (Emydidae). Journal of Experimental Zoology **220**:353–360.
Roff, D. A. 1992. The evolution of life histories. Theory and analysis. Chapman & Hall, New York, New York, USA.
Rohlf, F. J., and D. E. Slice. 1999. BIOMstat. Statistical software for biologists, version 3.2. Exeter Software, Setauket, New York, USA.
SAS. 1995. JMP. SAS Institute, Cary, North Carolina, USA.
Schwarzkopf, L., and R. J. Brooks. 1985. Sex determination in northern painted turtles: effect of incubation at constant and fluctuating temperatures. Canadian Journal of Zoology **63**:2543–2547.
Shine, R. 1999. Why is sex determined by nest temperature in many reptiles? Trends in Ecology and Evolution **14**:186–189.
Sokal, R. R., and F. J. Rohlf. 1995. Biometry. Third edition. W.H. Freeman and Company, San Francisco, California, USA.
Souza, R. R. d., and R. C. Vogt. 1994. Incubation temperature influences sex and hatchling size in the neotropical turtle *Podocnemis unifilis.* Journal of Herpetology **28**:453–464.
Spotila, J. R., L. C. Zimmerman, C. A. Binckley, J. S. Grumbles, D. C. L. A. Rostal, E. C. Beyer, K. M. Phillips, and S. J. Kemp. 1994. Effects of incubation conditions on sex determination, hatchling success, and growth of hatchling desert tortoises, *Gopherus agassizii.* Herpetological Monographs **8**:103–116.
Stearns, S. C. 1992. The evolution of life histories. Oxford University Press, New York, New York, USA.
Trivers, R. L., and D. E. Willard. 1973. Natural selection of parental ability to vary the sex ratio of offspring. Science **179**:90–92.
Valenzuela, N. 2001. Maternal effects on life history traits in the Amazonian giant river turtle *Podocnemis expansa.* Journal of Herpetology **35**, *in press.*
Valenzuela, N., R. Botero, and E. Martínez. 1997. Field study of sex determination in *Podocnemis expansa* from Colombian Amazonia. Herpetologica **53**:390–398.
Viets, B. E., M. A. Ewert, L. G. Talent, and C. E. Nelson. 1994. Sex-determining mechanisms in squamate reptiles. Journal of Experimental Zoology **270**:45–56.
von Hildebrand, P., N. Bermudez, and M. C. Peñuela. 1997. La Tortuga Charapa (*Podocnemis expansa*) en el Río Caquetá, Amazonas, Colombia. Aspectos de la Biología reproductiva y técnicas para su manejo. Disloque Editores, Santafé de Bogotá, Colombia.
Webb, G. J. W., A. M. Beal, S. C. Manolis, and K. E. Dempsey. 1987. The effects of incubation temperature on sex determination and embryonic development rate in *Crocodylus johnstoni* and *C. porosus.* Pages 507–531 *in* G. J. M. Webb, S. C. Manolis, and P. J. Whitehead, editors. Wildlife management: crocodiles and alligators. Surrey Beatty and Sons, Sydney, Australia.
Webb, G. J. W., and H. Cooper-Preston. 1989. Effects of incubation temperature on crocodiles and the evolution of reptilian oviparity. American Zoologists **29**:953–971.
Wibbels, T., J. J. Bull, and D. Crews. 1994. Temperature-dependent sex determination: a mechanistic approach. The Journal of Experimental Zoology **270**:71–78.
Wibbels, T., D. Rostal, and R. Byles. 1998. High pivotal temperature in the sex determination of the olive ridley sea turtle, *Lepidochelys olivacea,* from playa Nancite, Costa Rica. Copeia 1998:1086–1088.
Wilhoft, D. C., E. Hotaling, and P. Franks. 1983. Effects of temperature on sex determination in embryos of the snapping turtle, *Chelydra serpentina.* Journal of Herpetology **17**:38–42.
Yntema, C. L. 1968. A series of stages in the embryonic development of *Chelydra serpentina.* Journal of Morphology **125**:219–252.
Yntema, C. L. 1979. Temperature levels and periods of sex determination during incubation of eggs of *Chelydra serpentina.* Journal of Morphology **159**:17–28.