Field Study of Sex Determination in Podocnemis expansa from Colombian Amazonia

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FIELD STUDY OF SEX DETERMINATION IN
PODOCNEMIS EXPANSA FROM COLOMBIAN AMAZONIA

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ABSTRACT: We studied the relationship between fluctuating temperatures in the field and sex ratios of the giant river turtle (*Podocnemis expansa*) obtained from nests incubated in seminatural conditions using a stepwise logistic regression model. The number of hours above 31 C and mean temperature above 31 C during day 29–30 were the two parameters that best explained sex ratios. We also monitored incubation temperatures of natural nests. Mean incubation temperature and the combination of mean and variance were not predictors of sex ratio. We found a beach effect on sex ratios and incubation time. Natural nests hatched earlier than seminatural nests. Length of the incubation period was negatively correlated with degree days and mean incubation temperature in seminatural nests but not in natural nests. We found no evidence of an influence of length of the incubation period on sex ratio. Our results support current conservation practices in the study region.

Key words: *Podocnemis expansa*; Turtles; Sex determination; Incubation temperatures; Conservation; Colombia; Amazonia

NUMEROUS studies have shown the occurrence of various mechanisms of sex determination within the order Testudines, and in reptiles in general; these mechanisms are comprised by two major categories: genotypic sex determination (GSD) and environmental sex determination (ESD) (Bull, 1980; Ewert and Nelson, 1991; Ewert et al., 1994; Janzen and Paukstis, 1991; Lang and Andrews, 1994; Viets et al., 1994; Wibbels, 1994). Temperature-dependent sex determination (TSD) is the most common of the environmental mechanisms found in the turtle species studied so far (reviewed by Ewert et al., 1994, and Ewert and Nelson, 1991). The critical temperature represents the point where males and females are produced in a ratio of 1:1 and is the inflection point in the sex ratio curves (Mrosovsky and Pieau, 1991). This reversal of sex ratio occurs within a narrow range of just 1 C or 2 C, which is called the transitional range (Ewert and Nelson, 1991). The temperature-sensitive period (TSP) is the stage during which sex is determined (Mrosovsky and Pieau, 1991) and is found in the second third of the incubation pe-

riod of turtles and other reptiles with TSD (Bull, 1983). The exact mechanisms by which incubation temperature affects sexual determination are still unknown.

Podocnemis expansa exhibits temperature-dependent sexual determination (Alho et al., 1985; Lance et al., 1992), and current data suggest that the species is TSDIa (Ewert and Nelson, 1991); that is, females are produced at high temperatures and males at low ones. *Podocnemis unifilis* also exhibits TSDIa (Souza and Vogt, 1994). Gonads differentiate during days 25–35 of incubation in *P. expansa* (M. J. Mora, unpublished data). This is within the second third of the 60 day incubation period in the population inhabiting the Middle Caquetá River, Colombian Amazonia. External temperatures are also known to affect metabolic rates of reptiles (Bull and Vogt, 1981; Zug, 1993), and constant incubation temperature regimes are negatively correlated with length of the incubation period (Mrosovsky and Provancha, 1992; Thompson, 1988b). Thus, a relationship between sex ratio and length of the incubation period is expected in natural conditions as well.

Here we report on the influence of incubation temperature on sex ratios of

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hatchling *P. expansa* from the Middle Ca-
quetá River, Colombian Amazonia, pro-
duced under seminatural, natural, and ar-
tificial conditions. We describe sex-deter-
mination in the field based on these data
which comprise (1) parameters of temper-
ature that explain sex ratio, (2) description
of the thermoinfluential period, and (3)
the effect of incubation time. We also re-
port the effect of seminatural and natural
incubation on sex ratios, hatching rates,
and incubation time. Finally, the implica-
tions of these data for conservation prac-
tices will be discussed.

MATERIALS AND METHODS

The data on artificial incubation came
from a laboratory study performed during
1991. Eggs from four different nests laid
the night before were collected from the
bank of the Middle Caquetá River and
were transported to Bogotá. Two or three
days after oviposition, eggs from each nest
were assigned to four incubators set at
26.5 C, 28.5 C, 30.5 C, and 32.5 C (±0.5
C). Humidity was maintained at similar
levels in all four incubators, although no
exact measurement of water potential was
taken.

The remaining data come from a study
on natural and seminatural incubation
conducted during the reproductive season
1993–1994. Natural and transported nests
came from three different beaches along
the Middle Caquetá River: the Yarumal,
the Centro, and the Guadual beaches.
From each of the three beaches, we chose
and transported nests laid the night before
so that all the nests were of the same,
known age. We constructed seminatural
nests as 4 × 2 m wood boxes divided into
eight 1 × 1 × 1 m compartments filled
with sand. Each 1 m³ compartment housed
the eggs of just one clutch. From each
clutch, we used half the number of eggs
(approximately 40). Sand used in the in-
cubation of all transported nests came
from a single beach. We placed all nests at
approximately the same depth within the
compartments. These boxes were located
on top of a high beach to avoid the danger
of a sudden increase in water level, which
could drown the eggs. A total of 30 clutch-

es, 10 per beach, were incubated in this
way. On the same three beaches, an ad-
ditional 15 clutches were monitored where
they were laid, five per beach.

We recorded temperatures within nests
daily at 0700, 1300, and 1900 h, as well as
every hour (0100 h to midnight) one day
per week. From day 25 until day 35 of in-
cubation, we recorded temperatures every
other hour. Temperature records were
taken inside the egg chamber with a ther-
mometer accurate to ±0.1 C. We removed
hatchlings from seminatural and natural
incubation conditions from the nests after
hatching but before they emerged by
themselves. At least two nests from each
treatment (seminatural and natural incu-
bation), and each beach, were chosen to
determine sex-ratios (% of females) among
all hatchlings. In addition, we sexed all
hatchlings from the artificial incubation ex-
periment in Bogotá. This was performed 3
mo after hatching. We determined sex by
means of radioimmunoassay (RIA) of tes-
tosterone levels in the blood of the young
turtles 4 h after injection with FSH, which
stimulated higher than normal levels of
testosterone (Lance and Callard, 1979;
Lance et al., 1992; Owens et al., 1978).
This technique had been used and stan-
dardized for *P. expansa* in a previous study
(Lance et al., 1992) using 178 hatchlings,
which included the hatchlings from the ar-
tificial incubation reported here, plus
hatchlings collected from the wild along
the Middle Caquetá River. In that study,
comparisons of sex determination by RIA,
gonadal inspection, and histological dissec-
tion of the same individuals demonstrated
that the RIA method gave a perfect
(100%) match with actual sex.

Temperatures of seminatural nests were
used for the regression analysis with sex
ratios. We divided the data from days
25–35 into daily groups, and their relation-
ship with sex ratio was determined using a
stepwise logistic regression (Sokal and
Rohlf, 1995; SAS version 6.11, 1994). This
was done to determine which days ex-
plained most of the variance in sex ratios.
One of the parameters of temperature
tested in the regression analysis was the
number of hours >31 C for each of those

TABLE 1.—Sex ratios, incubation time, and temperature of seminatural and natural nests.

Nest	Beach	Treatment	Incubation time	% Female	n	No. h day 29–30 with T° >31 C	Mean T° >31 C day 29–30
S1	Guadual	Seminatural	73	90.3	22	3	33.2
S2	Guadual	Seminatural	77	73.9	23	1	31.8
S7	Guadual	Seminatural	75	73.3	23	1	31.5
S8	Guadual	Seminatural	74	75.0	16	3	31.3
S15	Yarumal	Seminatural	75	100.0	43	7	36.2
S16	Yarumal	Seminatural	75	100.0	12	7	35.9
S23	Centro	Seminatural	82	93.3	30	7	35.7
S25	Centro	Seminatural	82	100.0	20	7	34.9
S30	Centro	Seminatural	83	100.0	17	7	35.1
G6	Guadual	Natural	54	88.1	42		
G7	Guadual	Natural	62	95.7	23		
Y23	Yarumal	Natural	62	96.3	27		
Y38	Yarumal	Natural	69	94.7	19		
C7	Centro	Natural	66	100.0	28		
C13	Centro	Natural	62	88.2	34		

days. Mean temperatures >31 C were also tested in the regression analysis with sex ratio. Given that we took records for some nests on alternate days than others, the daily temperature records were classified as two-day periods: days 25–26, 27–28, 29–30, 31–32, and 33–34.

We calculated the relationship between sex ratio and length of incubation period using logistic regression as well. We calculated the correlation between mean incubation temperature and length of the incubation period, and the correlation between degree days and incubation time. We defined degree days (DD) as the heat-units accumulated above a threshold temperature (Baskerville and Emin, 1969). In this case, we calculated DD as the product of the number of days during which mean temperature was >28.5 C (below which no hatching was obtained under laboratory conditions in 1991) and the mean of the temperatures over those days, following the description made by Schwarzkopf and Brooks (1985). A nested ANOVA (Sokal and Rohlf, 1995) was performed to detect differences in sex ratios and length of incubation period between nests in natural beaches and those incubated in the seminatural nests, as well as to detect any beach effects.

RESULTS
Artificial Incubation

Incubators produced 100% males at 30.5 C and 69.7% males at 32.5 C. All em-bryos incubated at 26.5 C died before 10 days of development, and all at 28.5 C died before 25 days of development.

Sex Ratios from Natural versus Seminatural Incubation

Overall hatching rates were 95% under seminatural and natural (though protected) conditions. The sex ratios reported here (Table 1) correspond to all surviving hatchlings (some hatchlings were lost to predation by domestic dogs before sexing, but it was assumed that this predation was sex-independent and thus would not bias the sex ratios). Results of the nested ANOVA showed that there was no significant difference between sex ratios obtained from nests under seminatural incubation and those under natural incubation, which were not removed from where they had been laid by the females ($P > 0.05$). However, a beach effect was present ($P < 0.05$) (Table 2). Nests from Guadual beach showed a significantly smaller proportion of females than Yarumal beach ($t = 3.80$, $P < 0.005$) and Centro beach ($t = 3.66$, $P < 0.005$), but no significant difference was found in the sex ratios between Yarumal beach and Centro beach ($t = 0.35$, $P > 0.05$).

Incubation Temperature and Sex Ratios in the Field

Daily mean temperatures for all nests monitored in the field ranged from 26–31

TABLE 2.—Nested ANOVA of sex ratios and incubation time.

Source of variation	df	SS	MS	F_s	P
Sex ratio					
Between incubation conditions	1	62.5	62.5	0.27	>0.05
Among beaches	4	929.1	232.3	6.18*	<0.05
Within beaches	9	338.0	37.6		
Incubation time					
Between incubation conditions	1	792.1	792.1	18.0*	<0.005
Among beaches	4	175.6	43.9	5.3*	<0.025
Within beaches	9	73.9	8.2		

* Significant difference.

C. Sex ratio of all field nests ranged from 73.3–100% females. Of the number of hours >31 C during the critical period (days 25–35), only those from day 29–30 (Table 1) were included in the model by the logistic regression with stepwise procedure (P = 0.0001). Under this model, day 29–30 explained most of the variance in sex ratios, and no other variable (number of hours >31 C for any other day) contributed significantly when added to the model (Table 3). We also analyzed the influence of the mean of the temperatures >31 C on sex ratio by means of the stepwise logistic regression. Again, only the mean temperature >31 C for the day 29–30 was incorporated in the model (P = 0.0001). In order to assess whether some other variables other than those from day 29–30 had an indistinguishable fit to the data that could be obscured by the stepwise procedure, we calculated the logistic regression of each variable independently. All other variables showed a significantly worse fit to the data, leaving those from day 29–30 as best predictors of sex ratio.

Length of Incubation Period

Mean length of the incubation period was 77.3 days for seminatural nests, 62.5 for natural nests, and 71.4 days when combined. The nested ANOVA showed significant differences between length of the incubation periods in natural and seminatural nests (P < 0.005). A beach effect was found in the same analysis (P < 0.025) (Table 2). Nests from Centro beach showed significantly shorter incubation times than nests from Guadual beach (t = 3.36, P < 0.005) and Yarumal beach (t = 2.46, P < 0.025), but no differences were found between Guadual and Yarumal beaches (t = 0.58, P > 0.05). The effect of incubation conditions explained 82% of the variation in incubation time while the beach effect explained 11.5%. We could not find evidence of a relationship between length of the incubation period and sex ratio with our field data. No significant correlation was found between mean T° over the entire incubation period and incubation time for natural nests nor for the combined data from natural and seminatural nests, but it

TABLE 3.—Logistic regression with stepwise procedure; analysis of maximum likelihood estimates.

Variable	df	Parameter estimate	Standard error	Wald χ^2	P χ^2
Number of h > 31 C day 29–30					
Coefficient of H_{29} (b)	1	0.49	0.12	16.85	>0.0001
Intercept	1	0.46	0.38	1.42	>0.23
Mean > 31 C day 29–30					
Coefficient of M_{29} (b)	1	0.71	0.17	16.58	>0.0001
Intercept	1	−21.42	5.66	14.31	>0.0002

No other variable met the 0.05 criterion to be incorporated into the model.

was significant for the seminatural nests alone ($r = -0.74$, $P < 0.05$). However, a significant, negative correlation was found between DD and length of the incubation period ($r = -0.85$, $P < 0.05$).

DISCUSSION

Incubation Temperature and Sex Ratios

According to our preliminary laboratory data, pivotal temperature in *Podocnemis expansa* must be >32.5 C (30.3% females were produced at that temperature) but probably <34.5 C (Alho et al., 1985). If the critical temperature lies between 32.5 C and 34.5 C, it would mean that *P. expansa* has the highest pivotal temperature reported to date for any species of turtle (Ewert et al., 1994). Females of this species lay their eggs on the open, sunny parts of the beach, where temperatures get very high. Sudden increases in water level occur frequently during the middle or the end of the reproductive season (von Hildebrand et al., 1988) and may select for rapid development of the embryos. A high pivotal temperature in this species could then be explained as a compromise between nesting in hot places for rapid development (Ewert et al., 1994) and the production of a less biased sex ratio.

Bull (1985) suggested that with variable field data one must first identify the measures of temperature that associate with sex-ratio, and then find the time interval that shows the strongest association between sex-ratio and that measurement. This justified the subdivision of temperature data into weekly units, which were tested independently of each other for a correlation with sex ratio (Bull, 1985). Because our temperature records showed a daily pattern [as given by a test of autocorrelation of the time series data of temperature for each nest ($P < 0.05$)], we divided the data from days 25–35 into daily groups. In the studies conducted so far, association between parameters of temperature and sex ratios have been calculated using parametric correlation. Because sex ratio is a binomial variable, and thus contains non-normally distributed standard errors, associations based on parametric

correlations may not meet the statistical assumptions of the test. Therefore, logistic regression is a more appropriate mathematical model to analyze such data (Sokal and Rohlf, 1995) and to find the association between sex ratio and one or more independent variables. One of the parameters of temperature tested in the regression analysis was hours >31 C for each of those days. This temperature was chosen because during the 1991 laboratory study at constant temperature regimes, 100% males were produced at 30.5 C and 69.7% males were produced at 32.5 C, suggesting that temperatures >30.5 C were somehow responsible for the production of females. In addition, we also tested for the association between sex ratio and mean temperatures >31 C, because the time spent at a temperature 1 C above a threshold will not be equivalent to the same time spent at a temperature 5 C above that threshold (Georges, 1989). Our results show that the number of hours >31 C and the mean temperature >31 C during the day 29–30 explained most of the variance in sex ratios. Georges (1992) also found that temperature experienced by nests in the field during only two incubation days best explained the sex ratios produced by *Carettochelys insculpta* (thermal scores during days 30 and 35 of incubation). The positive relationship found in our study is consistent with both laboratory data and some observations at the Trombetas station in Brazil (Alho et al., 1985), where higher proportions of females were produced at higher temperatures. This, however, is a surprising result and is in contrast with the thermosensitive period reported for *Podocnemis unifilis,* which extends for more than the second third of incubation (Souza and Vogt, 1994). We have defined day 29–30 as the thermoinfluential period, because TSP has been defined as the time interval of incubation when temperatures affect sex ratio, and we cannot rule out minor influences of days different from day 29–30. These results would predict a male biased sex ratio from nests that do not experience temperatures >31 C and with a mean temperature <31 C during days 29–30 of incubation (or the equivalent de-

velopmental stage). Further work is necessary to test whether the thermoinfluencial period (i.e., the period that statistically best explains sex ratios) corresponds to the TSP for the species or not. It is also necessary to validate the biological significance of such a short thermoinfluencial period found in this study and the predictions that derivate from it. Complete temperature records as well as developmental series are essential to answer these questions properly.

Some studies conducted in the field have found that mean and variance of incubation temperatures or hours above a critical temperature during the critical period could be used as predictors of sex ratios (Bull 1985; Bull and Vogt, 1979; Morreale et al., 1982; Mrosovsky and Provancha, 1992; Mrosovsky et al., 1984, 1992; Pieau, 1982; Souza and Vogt, 1994; Spotila et al., 1987; Standora and Spotila, 1985; Wilhoft et al., 1983). However, it has also been suggested that mean and variance of temperatures in the field are not the best predictors of sex ratios (Georges, 1989; Schwarzkopf and Brooks, 1985; this study). Georges (1989) developed a mathematical model to analyze data on fluctuating incubation temperatures provided that variances are homogeneous over the incubation period. There are three main reasons why the mathematical model developed by Georges (1989) is inappropriate for our data. First, incubation temperatures within nests in the field fluctuate daily in such a way that variances are not constant, violating one of the model's assumptions. Second, the pivotal temperature has not yet been determined for *Podocnemis expansa,* and Georges' (1989) model considers the proportion of development above and below the critical temperature as the major factor responsible for female and male biased sex ratios in the field. However, even without knowing the exact value of the critical temperature for *P. expansa,* it is worth noting that daily and overall mean temperatures monitored in field nests were below the range comprising the presumed critical temperature (32.5–34.5 C), which according to Georges (1989) should produce only males. Our

data from the field covered the upper range of sex ratios (70–100%). Georges's (1989) model also failed to predict sex ratios of *P. unifilis* in the case reported by Souza and Vogt (1994).

Despite the statistical inconveniences mentioned before, we calculated the correlation between sex ratios and mean temperature, and the combination of mean and variance, to compare our data with the literature. We found no significant correlation between sex ratio and any of these variables. This result indicates that mean temperature or the combination of mean and variance (during the entire incubation period or during the critical period) were not predictors of sex ratio in our study ($P > 0.05$). On the other hand, significant product moment correlations were found for the day 29–30 between number of hours when nest temperatures remained >31 C (or the mean of those temperatures) and sex-ratio ($r = 0.92$ and $r = 0.95$ respectively, $P < 0.001$ in both cases). Thus, the results of this study would have been the same had correlation analysis been used. Other factors that might account for variance in sex ratios from natural nests are the date of oviposition, nest substrate (and humidity), nest depth, position of the eggs within the nest, and metabolic heat (Souza and Vogt, 1994; Thompson, 1988a). In our study, the first three factors were eliminated, because nests from each beach were of the same and known age, the sand used for the seminatural nests came from the same beach, and the eggs were placed at approximately the same depth within each compartment. Metabolic heat must be an important factor for large clutches (Thompson, 1988a) as those of *Podocnemis expansa.* We divided the clutches and placed half the eggs in each seminatural nest. It is not known how this reduced the effect of metabolic heat, but the number of eggs did not affect sex ratios in a study on *Podocnemis unifilis* (Souza and Vogt, 1994). Additionally, the differences in location of the seminatural nests with respect to each other could also account for part of the temperature variation recorded. The position of the eggs within the egg chamber is yet another fac-

tor that could not be accounted for, because temperature records were taken at just one position within the egg chamber. Finally, we did not measure or control the water potential of the seminatural nests. Differences of water potential among nests could have existed, which might explain some of the variation in sex ratio production because the hydric environment may affect sex determination (Gutzke and Paukstis, 1983; Paukstis et al., 1984; Thompson, 1988*b*).

Nests within each beach used in this study were laid on the same night. Thus, nests within beaches might have experienced more similar temperature schedules than nests from different beaches. The synchrony of development experienced by nests coming from the same beach generated in this way may be the cause of the beach effect on sex ratio found in our data, as well as the beach effect found on incubation times.

Length of the Incubation Period

Natural nests were found to have shorter incubation periods when compared to seminatural ones. Higher temperatures are expected to increase metabolic rates (Ackerman, 1977; Zug, 1993) and thus shorten incubation period. In our laboratory experiments, we observed a negative relationship between incubation temperatures and length of the incubation period. This has also been reported for other species (Jeyasuria et al., 1994). Eggs of *P. expansa* incubated at 34.5 C and 33.4 C hatched after 50 days of incubation, those at 32.5 C hatched after 57 days, and those incubated at 30.5 C hatched after 62 days. In our study of seminatural and natural nests, a significant negative correlation was found between DD and length of the incubation period. However, mean temperature was not a good predictor of incubation time. Length of the incubation period was not predictor of sex ratio. It has been stated that higher temperatures are responsible for higher proportion of females. It was thus expected that shorter incubation periods at higher temperatures will produce a higher proportion of females. Our results did not support such expecta-

tions and suggest that additional variables, such as water potential, need to be controlled for or measured in the future in order to account for variation of sex ratios in the field. Further studies are necessary to account for the discrepancies that we found.

Mean length of the incubation period recorded for natural nests (62.5) is longer than that reported by Alho et al. (1985) for the population of *Podocnemis expansa* in Brazil. This difference with the data reported for Brazil may be due to climatic differences between both regions or to geographical differentiation of the populations from Colombia and Brazil.

CONCLUSIONS

We have described the sex determination of *Podocnemis expansa* during a field season in the Colombian Amazonia. The parameters that best explained the sex ratios produced are (1) the number of hours >31 C and (2) mean temperatures >31 C during the day 29–30 of incubation. Further work is necessary to determine the critical temperature and to define conclusively the thermosensitive and thermoinfluencial periods in terms of development. It is also necessary to corroborate the validity of 28.5 C and 31 C as the developmental and female-inducing thresholds respectively. Existing models did not explain the sex ratios found in our field study. New models that take into account fluctuating temperatures with heterogeneous variances need to be developed in order to account for TSD in the field. At the same time, it is essential for us to obtain complete temperature records in the field, together with data on additional variables such as water potential and development, in order to test such models.

Results concerning hatchling rates and the comparison of natural to seminatural sex ratios are encouraging with regards to conservation and management plans for this endangered species. Hatchling success was much higher in seminatural incubation conditions (95%) than in natural (unprotected) conditions (37%) (von Hildebrand et al., 1988). No significant difference in sex-ratio was found between nat-

ural and seminaturally incubated nests, which suggests the great potential of the seminatural incubation as part of the effort to increase population numbers of *P. expansa* without distorting natural sex-ratios.

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