Temperature-sex determination in *Podocnemis expansa* (Testudines, Podocnemididae)

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ABSTRACT. This study has been carried out at the central region of the Araguaia river on the border between the states of Goiás and Mato Grosso in the Brazilian Amazon Basin from September to December 2000. We recorded temperature fluctuation, clutch-size, incubation period and hatching success rate and hatchlings' sex ratio of five nests of *Podocnemis expansa* (Schweigger, 1812). Despite the relatively small sample size we infer that: a) nests of *P. expansa* in the central Araguaia river have a lower incubation temperature than nests located further south; however, incubation period is shorter, hatching success rate is lower and clutch-size is larger; b) *Podocnemis expansa* may present a female-male-female (FMF) pattern of temperature sex-determination (TSD); c) thermosensitive period of sex determination apparently occur at the last third of the incubation period; and, d) future studies should prioritize the relationship between temperature variation (*i.e.*, range and cycle) and embryos development, survivorship and sex determination.

KEYWORDS. TSD, thermosensitive period, sex determination, reproductive biology, turtles.

RESUMO. Determinação do sexo pela temperatura em *Podocnemis expansa* (Testudines, Podocnemididae). Este estudo foi realizado na região central do rio Araguaia, na fronteira entre os estados de Goiás e Mato Grosso, na Amazônia brasileira, durante os meses de setembro a dezembro de 2000. Além da variação da temperatura, foram avaliados o tamanho da ninhada, período de incubação, o sucesso de eclosão e a razão sexual dos filhotes recém eclodidos de cinco ninhos de *Podocnemis expansa* (Schweigger, 1812). Apesar do esforço amostral relativamente restrito, nós inferimos que: a) os ninhos estudados de *P. expansa* na região central do Rio Araguaia exibem temperaturas mais baixas do que ninhos mais ao sul, porém o período de incubação é menor, a taxa de eclosão é mais baixa e o tamanho de ninhada é maior; b) *Podocnemis expansa* pode mostrar um padrão fêmea-macho-fêmea (FMF) de determinação do sexo pela temperatura (TSD); c) o período termo-sensível para a determinação sexual aparentemente ocorre no último terço do período de incubação; e, d) futuros estudos devem priorizar a relação entre a variação da temperatura (*i.e.*, amplitude e frequência) e o desenvolvimento embrionário, sobrevivência e a determinação sexual dos filhotes.

PALAVRAS-CHAVE. TSD, período termo-sensível, determinação sexual, biologia reprodutiva, tartarugas.

The giant South American river turtle - *Podocnemis expansa* (Schweigger, 1812) - is one of the largest freshwater turtles of the world. It occurs in rivers and lakes of the Amazon and Orinoco river basins (Smith, 1979). The females reach 80 cm of carapace length and 60 kg of body-mass, whereas the males are smaller (Pritchard & Trebbau, 1984).

Decades of overexploitation resulted in dramatic population declines in many rivers of the Amazon basin (Smith, 1979). For this reason, the Brazilian government established a "ranching" program (*sensu* Hutton & Webb, 1992) based on the protection of nesting habitats, translocation of eggs, commercial use of captive-reared turtles, and release of hatchlings in the wild near their nesting areas of origin (FAO, 1988; IBAMA, 1989).

The manipulation of nests and eggs can affect the sex of embryos, since the species presents temperature-sex determination (TSD) (Alho *et al.*, 1985). For conservation purposes, in theory, a 1:1 sex ratio would be more convenient (Davenport, 1997). However, for commercial purposes ranchers could desire a female-biased population because females are larger than males, produce eggs, and a few males can inseminate a great number of females (Vogt, 1994).

Changes on the sex ratio of a population could in theory affect its dynamics within a few generations (Caughley, 1977). In addition, temperature of incubation can also affect other embryonic aspects in reptiles such as incubation period, integument pigmentation and post-hatching growth rate (Joanen & McNease, 1987; Deeming & Ferguson, 1989; Webb & Cooper-Preston, 1989). For these reasons, this study aims to determine the temperature of incubation in natural nests of the species and its pattern for the sex determination of embryos.

MATERIAL AND METHODS

We conducted this study in the central region of the Araguaia river basin on the border between the states of Goiás and Mato Grosso in the Brazilian Amazon Basin from September to December 2000. We sampled five nests distributed in four sand beaches at both margins of the Araguaia river (13°21'57.6"S, 50°39'05.7"W; 13°30'12.4"S, 50°44'12.4"W; 13°23'32.3"S, 50°40'12.8"W; 13°25'11.5"S, 50°39'55.6"W). The following nest characteristics were taken: egg-chamber depth and width, distance from the water and sediment type (according to Bonach *et al.*, 2003, 2006, 2007). Fieldwork was undertaken 7:00 to 9:30 AM or 4:00 to 6:30 PM in order to avoid egg desiccation. We implanted a data-logger Hobo TBI32-20+50 Onset (precision ± 0.1 C) in the middle of the top layer of the egg-chamber of each nest, approximately 25 to 49 cm beneath the surface (according to Bonach *et al.*, 2006), at day 1 of the incubation period. The data-loggers were programmed to store temperature every other hour during the whole incubation period.

This interval was considered short enough to prevent undetected peaks in temperature. They were tied by a thin rope to a numbered stick fixed in the ground above the nest. All nests were fenced for protection against predators and checked at day 45 of incubation in order to evaluate the development of embryos.

Besides temperature fluctuation we recorded clutch-size, incubation period and hatching success rate of all nests. Sex ratio of all hatchlings (n=386) was determined for the five clutches. Regression equations were established between the percentage of females per clutch and the average temperature for the three thirds of the incubation period (i.e., days 1 to 20, 21 to 40, 41 to hatching), both treated as continuous variables (SOKAL & RHOLF, 1995). In order to be sexed, hatchlings were sacrificed in sulphuric ether chambers at the age of 30 days after hatching. Gonads were extracted, fixed in formaldehyde 10%, included in paraffin and transversally cut in order to prepare histological slides. Sex identification followed the description of MALVASIO *et al.* (1999) (Figs 1, 2). At the time of the analyses, non-lethal sexing techniques such as hormone level (LANCE *et al.*, 1992), geometric morphometrics (VALENZUELA *et al.*, 2004) and laparoscopy (KUCHLING, 2006) were either considerably less accurate or not efficiently applicable to hatchlings this small (< 70g).

RESULTS

The mean incubation temperature of the nests was 30°C ± 1.1 (range: 17.8-46.3) n = 5, standard error of mean (sem) = 0.5° C. The mean clutch-size was 98.8 eggs ± 4.0 (94-104) n = 5, sem = 1.8. The mean hatching success rate was 78.2% ± 21.4 (42.6-99.0) n = 5, sem = 9.6%. The incubation results are presented in table I, and the histological slides of gonads of females and males are shown in figures 1 and 2, respectively.

The following regression equation has been successfully established between the average temperature of incubation between day 41 and hatching and the percentage of females in the clutch (Fig. 3): $y = 9742 - 687.7x + 12.19x^2$; where: y = % of females; x = mean temperature of incubation (°C) between day 41 of incubation and hatching); $r_{ad}^2 = 97.7\%$; P = 0.011; df = 4. However, there was no correlation between the following variables: mean temperature of incubation

between day 1 and day 20 and percentage of females (P = 0.536, df = 4); mean temperature of incubation between day 21 and day 40 and percentage of females (P = 0.268, df = 4); mean temperature of incubation (°C) and incubation period (days) (P = 0.288, df = 4); mean temperature of incubation (°C) and eclosion rate (P = 0.937, df = 4); minimum temperature of incubation (°C) and eclosion rate (P = 0.649, df = 4); variance of incubation temperature and incubation rate (P = 0.097, df = 4); and variance of incubation temperature and eclosion rate (P = 0.716, df = 4) and incubation period (days) and eclosion rate (P = 0.716, df = 4).

DISCUSSION

Besides daily fluctuations, temperature variation is possibly also related with sand physical characteristics such as grain size distribution and thermal conductivity in *P. expansa* nests (SOUZA & VOGT, 1994). The optimum thermal range for the incubation of chelonian eggs is between 29°C and 35°C (EWERT, 1985). In this study, the average temperature of the nests is in this range. However, we found mean, minimum and maximum temperatures below those reported for the species at Araguaia National Park in the southern Araguaia river 33°C to 35°C, 25.8°C and 39.1°C, respectively (A. Malvasio, pers. observ.). VON HILDEBRAND *et al.* (1988) report an average temperature of 28°C 15 cm deep for *P. expansa* nests in Colombia. However, SPOTILA *et al.* (1987) did not find significant thermal differences according to different depths in *Chelonia mydas* (Linnaeus, 1758) nests.

SPOTILA *et al.* (1987) reported a much smaller range of daily thermal fluctuation (1.5°C) in *C. mydas* nests than we found for *P. expansa* in this study (28.5°C). This adds some complexity to the study of the role of temperature of incubation on sex determination of freshwater turtles, in such a way that not only mean temperatures should be considered but also extreme temperatures eventually experienced by the embryos and – possibly even more important – the range and frequency of daily thermal variation as stated by GEORGES *et al.*, (1994) for *Caretta caretta* (Linnaeus, 1758). However, the relationship between incubation temperature daily fluctuation and embryos' sex determination in turtles is still unclear (VALENZUELA, 2001).

Tab. I. TSD in *Podocnemis expansa* (Schweigger, 1812) in central Araguaia river, border between the states of Goiás and Mato Grosso in the Brazilian Amazon Basin, from September to December 2000 (n = 5) (standard error of mean < 0.15°C for all average temperatures below).

Nest	Clutch-size	Average temperature (°C) (Day 1 to hatching)	Average temperature (°C) (Day 1 to 20)	Average temperature (°C) (Day 21 to 40)	Average temperature (°C) (Day 41 to hatching)	Incubation period (days)	F (%)	M (%)
11	96	28.4	28.3	29.8	26.5	53	81.9	18.1
25	104	31.2	30.9	32.3	30.1	58	89.1	10.9
37	101	29.8	30.5	31.7	28.2	72	44.3	55.7
38	94	30.1	29.9	31.5	29.1	61	58.6	41.4
39	99	30.7	30.8	32.0	29.0	56	52.6	47.4

The analysis of variance of the coefficients generated by the Fourier Analysis (Bloomfield, 1976) can be useful in future studies, helping detect for instance eventual intraspecific differences of the pivotal temperature, as observed by Chevalier *et al.* (1999) for Atlantic and Pacific nesting populations of *Dermochelys coriacea* (Linnaeus, 1766). However, this approach needs specific experimental design where both frequency and amplitude of temperature variation can be tested as distinct dependent variables.

The hatching success rate found in this study was lower than previous reports for the species in northern Amazon (88.9 %) (Zwink & Young, 1990) and southern Araguaia river (92.5%) (A. Malvasio, pers. observ.). However, the great variation of the species nesting habitats mentioned above and the small sample size of this study prevent us to take definite conclusions about this. *Podocnemis expansa* occurs on a broad geographic area with a considerable heterogeneity of nesting habitats in space and time. Therefore, we can expect a great variation of nesting success from year to year on a micro- as well as on a macrogeographic scale (Verdade *et al.*, 2002; Villela *et al.*, 2008).

Although we had lower temperatures of incubation than those recorded by A. Malvasio (unpubl. data), the average period of incubation in both studies were quite similar (58.2 days in this study and 59 days, A. Malvasio, unpubl. data) and intermediate in relation to others (Alho *et al.*, 1979: 48 days; Von Hildebrand *et al.*, 1988: 67.2 days).

The average clutch-size found in this study was slightly larger than those described for other areas
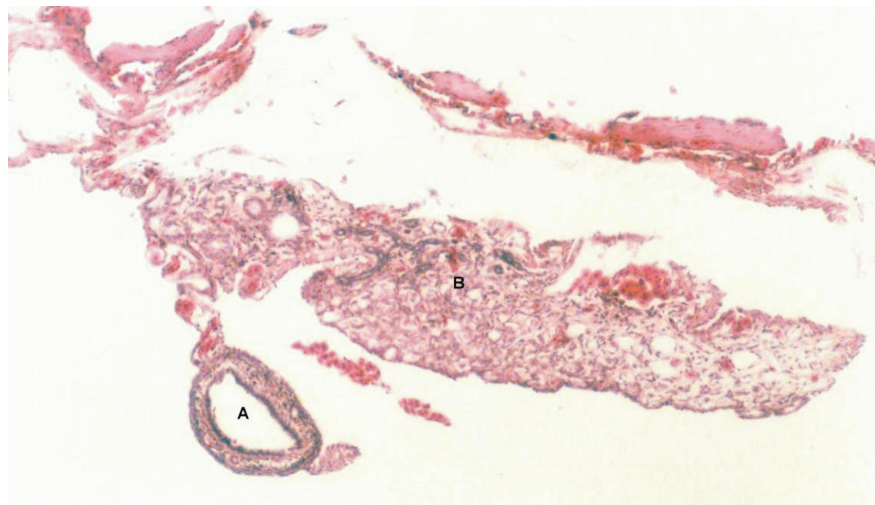

Fig. 1. Ovary histological slide of *Podocnemis expansa* (Schweigger, 1812) hatchling (A, oviduct; B, ovary). Coloration: Hematoxilin-eosin. Photomicroscope: 36x enlargement.
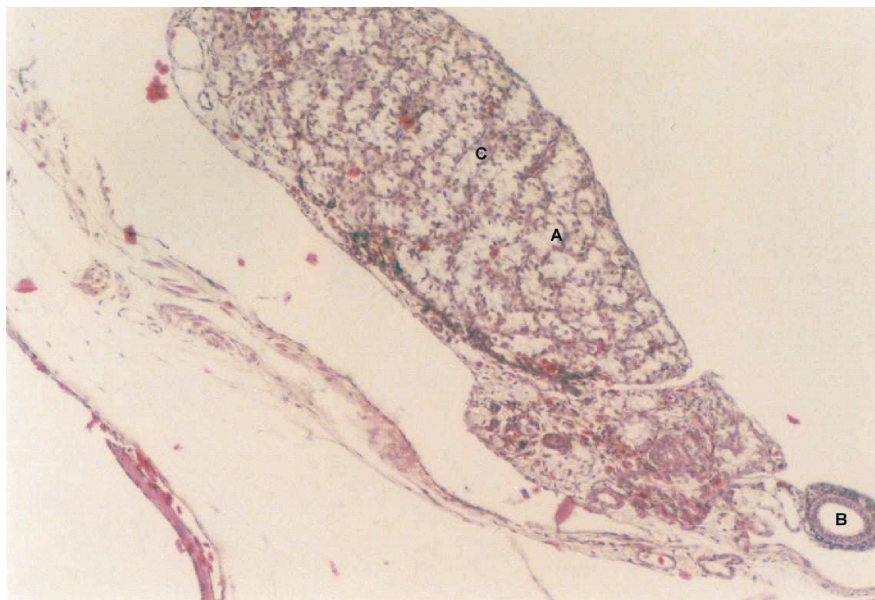

Fig. 2. Testicle histological slide of *Podocnemis expansa* (Schweigger, 1812) hatchling (A, vacuolized cells; B, deferens duct; C, testicle). Coloration: Hematoxilin-eosin. Photomicroscope: 36x enlargement.

within the species distribution: 91.5 eggs (Trombetas river, 1°20'S, 56°45'W, n=393) (ALHO & PÁDUA, 1982) and 95 eggs (Javaés river, 9°50' to 11°10'S, 49°56'W to 50°10'W, n=20) (A. Malvasio, pers. observ.). These differences can be related to the latitude (LITZGUS & MOUSSEAU, 2006). Oviparous reptiles usually present a positive correlation between female body-size and clutch-size (*e.g.* BONACH *et al.*, 2003, 2006, 2007; LARRIERA *et al.*, 2004; VERDADE, 2001). There can be both micro- and macrogeographic variation in females' body size (MOSKOVITS, 1988; BAUWENS & CASTILLA, 1998). Future studies on the allometry of reproduction might help us assess the female fecundity curve for a focused population and hence to establish simulation models of population fluctuation (ABERCROMBIE & VERDADE, 1995).

Hatchling turtles do not present an evident sexual dimorphism on their secondary sexual characteristics. Therefore, to sex hatchlings it is necessary to use more invasive techniques such as the determination of the testosterone seric level after FSH injection (VALENZUELA *et al.*, 1997), gonads anatomy (MALVASIO *et al.*, 1999, 2002), histological identification of the ovary and testis (DANNI & ALHO, 1985; MALVASIO *et al.*, 2002) and laparoscopy (VOGT, 1994). In this study we used the histological identification of the gonads with more than 99% of secure classification, by far more accurate than the other methods.

TSD has been previously described for *P. expansa* with males produced at lower and females at higher temperatures (MF pattern) (ALHO *et al.*, 1985; LANCE *et al.*, 1992; SOUZA & VOGT, 1994). However, VALENZUELA (2001) suggests the species can also present a FMF pattern, with females produced at the extreme temperature, what is corroborated by the present study.

The thermosensitive period for sex determination in chelonians is considered to be the second third of the incubation period (BULL, 1983). However, in the present study the thermosensitive period apparently occurred after the day 40 of incubation. Considering the average incubation period of 58 days, the thermosensitive
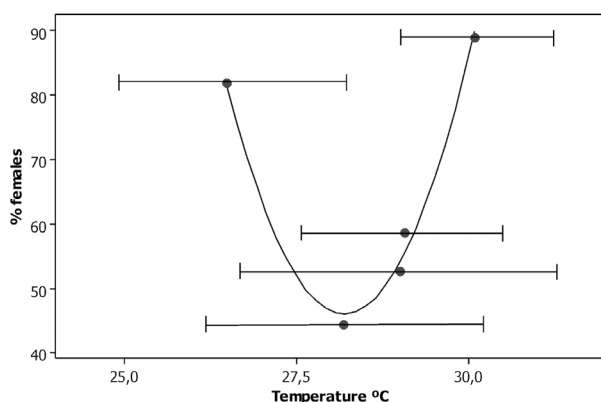
period for sex determination surprisingly seemed to occur at the last third. Some intraspecific variation can in theory occur, but its evolutionary implications are still undetermined and should be pursued in future studies. On the other hand, the implications of a late sex determination for ranching management programs are clear: sex ratio of hatchlings will be affected by early egg translocation. This should be a concern for managers, since in some conservation turtle projects, there are eggs transferences. At last, in this study we considered only mean temperatures, whereas extreme temperatures may eventually be biologically more relevant. For this reason, future studies should prioritize the relationship between temperature variation (*i.e.*, range and cycle) and embryos development, survivorship and sex determination.

Fig. 3. Relationship between the temperature of incubation [mean (dots) ± standard deviation (lines)] between day 41 of incubation and hatching and the ratio of females of *Podocnemis expansa* (Schweigger, 1812) at the central region of the Araguaia river on border between the states of Goiás and Mato Grosso, from September to December 2000.

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