NEST TEMPERATURE, INCUBATION TIME, HATCHING, AND EMERGENCE IN THE HILAIRE'S SIDE-NECKED TURTLE (*PHRYNOPS HILARII*)

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Abstract.—The nest dimensions, physical characteristics of the eggs, thermal exposure, incubation period, and hatchling emergence were investigated. A total of 12 nests were monitored: six (N = 50 eggs) in natural conditions and six (N = 28 eggs) in artificial conditions. Nests constructed by females have an aperture, a neck, and an incubation chamber with mean dimension of 143 by 126 mm. Eggs (N = 78) were characterized as spherical, 34 x 32 mm, with a calcareous shell and mean mass of 21.5 g. The incubation period varied from 157 to 271 days, in natural conditions, and from 130 to 191 days under artificial conditions; whereas, hatching success varied from 43 to 75%, and from 50 to 100%, respectively. The mean temperature inside the natural nests ranged from 24.2 to 27.3°C; whereas, under artificial conditions it ranged from 18.8 to 28.6°C. Significantly more hatchlings emerged from eggs incubated under artificial conditions than from natural nests.

Key Words.—Chelidae; development; emergence; freshwater turtles; hatching; incubation; nest temperature; *Phrynops hilarii*

INTRODUCTION

All chelonians are oviparous and lay their eggs in nests constructed by females in sandy substrates, or under dry leaves and detritus. Chelonians can build their nests in shady areas, or open areas with higher exposure to solar radiation (Ewert 1979). A combination of favorable location, substrate type (e.g., sandy, muddy, or decomposing vegetation), and egg depth can lead to an optimum environment for embryonic development. As chelonian eggs do not receive any direct parental care, embryonic development (i.e., incubation, hatching, and juvenile emergence) depends on the environmental conditions to which the eggs are exposed.

The thermal and moisture conditions of the nest are important for egg incubation, influencing the development rate, incubation time (Yntema 1978), and survivorship of the embryo (Packard and Packard 1988; Booth 1998). Moreover, thermal and hydric conditions determine sex in many chelonian species (Bull 1980; Ewert and Nelson 1991), play an important role in the size of neonates (Packard et al. 1987; Packard and Packard 1993), and on their locomotor development (Miller et al. 1987; Janzen 1995). Iincubation temperature, however, has a greater influence on species that lay eggs with a hard shell (Janzen 1993) than water content of the soil (Leshem and Dmi'el 1986). Humidity does not appear to affect the embryo or hatchling's metabolism in these species (Packard et al. 1979, 1981; Packard and Packard 1991).

Hilaire´s Side-necked Turtle, *Phrynops hilarii* (Fig. 1), is the second most abundant chelonian species in the delta of the Jacuí River of Brazil inhabiting lentic and lotic environments (Bujes and Verrastro 2008). In this region, nesting activity occurs from September to October and February to March, being associated with a minimum mean air temperatures of 26°C. Females produce 10–22 eggs with hard shells, and present daily bimodal nesting activity with peaks at sunrise and sunset (Bujes 1998). The objectives of our study were to examine the influence of soil temperature on the incubation period and on the phenotypic variation of the hatchlings; as well as, the hatching and emergence behavior from the nest in natural and artificial conditions, eliminating potential effects of the substrate humidity.

MATERIALS AND METHODS

We identified 18 nests of *Phrynops hilarii* and randomly selected six, which we monitored from

FIGURE 1. Female Hilaire's Side-necked Turtle, *Phrynops hilarii* (Testudines, Chelidae). (Photographed by Clóvis Bujes)

FIGURE 2. Aerial photography of the study area: Pintada Island, Rio Grande do Sul State, Brazil.

September 2004 to October 2005. The nesting site was located in a grassy area between two buildings of the Delta do Jacuí State Park, south of Mauá canal, on Pintada Island (30°01'52"S, 51°15'07"W), Porto Alegre, Rio Grande do Sul state, Brazil (Fig. 2).

We recorded air (measured about 100 mm from the surface of the nest), substrate, and nest chamber (i.e., among the eggs) temperatures immediately after oviposition using mercury thermometers (± 1°C). Total depth (from the surface to the end of the incubation chamber) and the diameters (highest vs. lowest) of the nests were measured with a measuring tape (± 1.0 mm). We removed eggs from the nest, which we counted, marked, and weighed using a digital scale (Giros PG-500®; total capacity of 500 g, ± 0.1 g). We also measured eggs (in their longest and shortest diameter) with a 200 mm Mitutoyo® caliper (± 0.05 mm).

We randomly chose 40% of the eggs from each nest and incubated them under artificial conditions. We returned the remaining eggs to the original nests, where they were replaced in the same order they were removed. We placed a silicon rod (20.0 cm in length x 0.5 cm in width) vertically, in the center of the incubation chamber among the eggs and the eggs were then reburied. To protect and facilitate the locating each nest, we surrounded the nest and the silicon bar by a plastic screen. We monitored the temperatures that the eggs were exposed to inside the nests, as well as soil and air temperature (at 100 mm above the surface) at three hour intervals for a period of 24 h every month during the first five months of the incubation period. Approximately 1 h before the beginning of each monitoring period, we carefully removed the silicon bar from the nest and in its place we inserted a 200 mm mercury thermometer. We could observe any hatchling movement inside the incubation chamber through the hole made by the silicon bar.

We placed eggs incubated under artificial conditions in coolers (1000 mm x 400 mm x 350 mm) with a substrate composed of two parts of equal volumes of vermiculite to one part of water by volume. We monitored coolers with eggs weekly and we recorded nest temperatures and egg status. When the substrate was dry, we added water to maintain substrate hydration. We defined hatching as the pipping of the eggs; whereas, emergence was defined as when hatchlings left the nest chamber.

We counted, weighed, and measured neonates, both in natural and artificial conditions. Measurements included straight-line carapace length (CL) and maximum width (CW), plastron length (PL) and width (PW), and shell height (SH; for details see Bujes 2008). After an observation period of approximately two weeks, we released the hatchlings in an area of lentic and vegetated waters, close to the nesting site.

We calculated the variation between mean egg mass and the variation among eggs from different nests with an Analysis of Variance (ANOVA). We used an F-test to calculate the difference between biometric data of the hatchlings hatched in natural and artificial conditions. We ran statistical tests using the computer program SPSS 11.0 for Windows. For each test, α = 0.05).

RESULTS

The dimensions of the egg chambers in the six nests varied from 110 to 149 mm ($\overline{x}$ = 126.33 mm, SD = 16.98, N = 6) in minimum width, and from 130 to 170 mm ($\overline{x}$ = 143.33, sd = 15.06, N = 6) in maximum width; the depth of the nests varied between 116 and 170 mm ($\overline{x}$ = 151 mm, SD = 18.71, N = 6). The last egg laid by a female was located between 30 and 75 mm below the surface ($\overline{x}$ = 53.83 mm, SD = 18.22, N = 6).

We characterized the eggs of *P. hilarii* as smooth, calcareous, and spherical ranging between 0.88 and 1.0 mm ($\overline{x}$ = 0.956, SD = 0.03, N = 78). The mean length of the larger axis of the eggs varied between 30.9 and 37.0 mm ($\overline{x}$ = 34.12 mm, SD = 1.13, N = 78); the smaller axis varied from 30.1 to 35.4 mm ($\overline{x}$ = 32.62 mm, SD = 1.31, N = 78), and the egg mass varied between 17.0 and 26.3 g ($\overline{x}$ = 21.47 g, SD = 2.1, N = 78). The mean egg mass was significantly different among nests (F = 73.47, df = 5, P < 0.001), and the variation of mass among eggs from the same nest was 0.77 g.

The incubation period of the eggs under natural conditions varied from 157–271 days ($\overline{x}$ = 221.5 days, SD = 49.33, N = 6); whereas, under artificial conditions, incubation time varied from 130–191 days ($\overline{x}$ = 167.8 days, SD = 23.51, N = 6). Hatching success varied from 43 to 75% (30 hatchlings out of 50 eggs) under natural conditions; whereas, in the laboratory, it varied from 50 to 100% (25 hatchlings out of 28 eggs; Table 1). There were four non-viable eggs with no visible signs of development.

The mean temperature inside nests varied from 24.2 to 27.3°C (Fig. 3). This was similar to the mean temperature of the substrate, which varied from 24.6 to 27.1°C, and to the air temperature that ranged from

TABLE 1. Number of eggs, number of hatchlings, hatching rate, and incubation and emergence periods in the nests of *Phrynops hilarii* from Brazil incubated in natural (N) and artificial (A) conditions.

| Nest	Oviposition date	Number of Eggs	Number of hatchlings	Hatching rate (%)	Incubation (days)	Emergence (days)
Ph1N	10/12/04	7	3	42.9	255	68 ± 3
Ph1A		4	4	100	186	~ 2
Ph2N	10/14/04	12	7	58.3	269	3
Ph2A		6	6	100	183	~ 3
Ph3N	10/21/04	7	4	57.1	189	5
Ph3A		5	4	80.0	164	~ 2
Ph4N	10/21/04	8	6	75.0	157	12
Ph4A		5	5	100	130	~ 4
Ph5N	09/14/04	8	5	62.5	188	4
Ph5A		4	4	100	153	~ 3
Ph6N	09/08/04	8	5	62.5	271	10
Ph6A		4	2	50.0	191	~ 1
Total		78	55			

23.4 to 25.3°C (Table 2). Thermal limits recorded in the nesting area varied from 9°C (soil surface) to 27°C (50 mm deep) between July and September 2004, and from 16°C (air) to 40°C (soil surface) between October and December of the same year. The mean temperature in the incubation chamber was 22.2°C (SD = 2.94, ranging from 18 to 30°C, N = 94).

Neonates hatched under laboratory experiments were larger than those hatched from natural nests (Table 3). All measurements between the two groups, including mass, were significantly different ($P < 0.001$), with exception of PW ($P = 0.016$).

Under natural conditions, neonates hatched at dusk (1800–2000) in 66% (4/6) of nests. Hatchlings emerged from one nest after 2200 because we found hatchlings at the nest site at 0600. In another nest, we could not accurately estimate the time of emergence,

because we discovered that the plastic screen had confined these emerged hatchlings three days after the last observation. This nest presented the longest time inside the nest after observing activity of hatchlings: a total of 68 days. We did not observe external yolk sacs on any of the emerged hatchlings (all hatchlings had flat plastrons).

Under laboratory conditions, all hatchlings emerged at night and had a visible external yolk sac. Most hatchlings (92%) were inactive and stayed semi-buried in the incubator vermiculite for approximately four days. During this period, the hatchlings absorbed the yolk while expanding their shells, losing the oval shape of the carapace and the folding of the plastron. After this period, all hatchlings increased activity. Hatchling emergence was synchronous under both natural and artificial conditions.
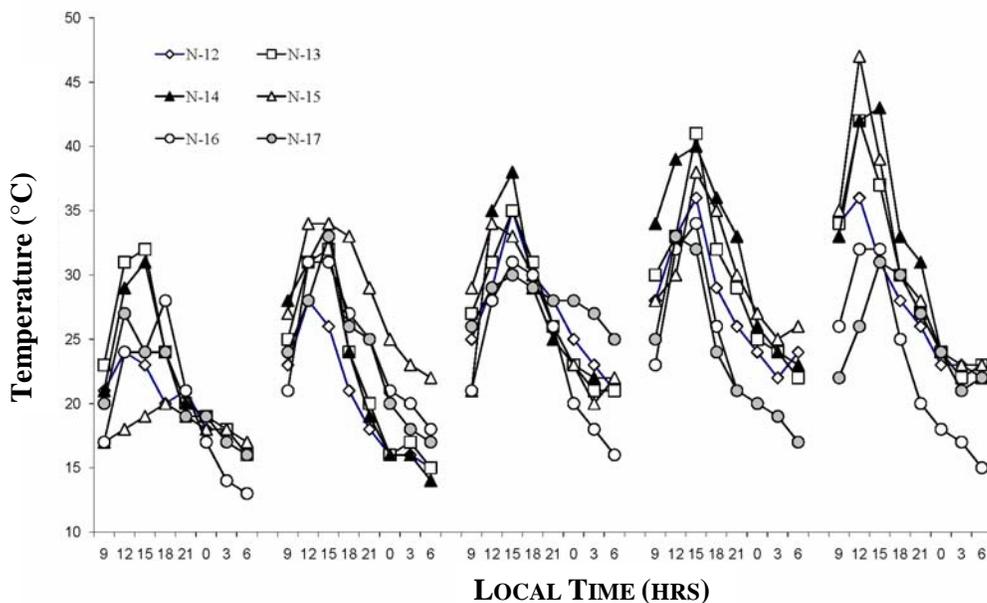
FIGURE 3. Representation of thermal variation observed in the nests of *Phrynops hilarii* in a 24-h period in the first five months of incubation in natural conditions in Pintada Island, Rio Grande do Sul state, Brazil.

TABLE 2. Mean temperatures (ºC), standard deviation (SD), extreme values, and sample size (N) of the air, substrate, and interior of the nests of *Phrynops hilarii* in Pintada Island, Rio Grande do Sul state, Brazil.

| Nest | Mean temperatures (ºC) | | | N |
	Air	Substrate	Nest	
1	23.4	24.6	24.2	40
	SD = 5.28, 13–33	SD = 5.62, 15–36	SD = 3.98, 17–30	
2	24.1	26.3	26.2	40
	SD = 5.31, 13–33	SD = 6.72, 15–42	SD = 3.49, 20–32	
3	25.3	27.1	26.1	40
	SD = 5.72, 13–37	SD = 7.73, 14–43	SD = 4.38, 18–35	
4	24.5	27.0	27.3	40
	SD = 4.44, 18–33	SD = 6.91, 17–47	SD = 4.41, 19–35	
5	23.4	25.2	25.8	64
	SD = 5.35, 14–33	SD = 6.31, 13–40	SD = 3.91, 19–36	
6	24.0	26.1	26.4	64
	SD = 5.43, 14–37	SD = 6.36, 12–42	SD = 3.64, 17–35	

DISCUSSION

The nesting activity, structure, and nesting site for *Phrynops hilarii* has been described by Bujes (1998). In that study, the mean nest depth recorded was 121.3 mm, varying between 100 and 150 mm. In the present study, the mean depth was 151 mm (ranging from 116 to 170 mm). The number of eggs in *P. hilarii* nests has varied from 1–23 eggs, which has been thought to be related to the size and physiological conditions of the female (Cabrera 1998). Astort (1984) suggested that the nests of this species may vary from 8–32 eggs. The mean number of eggs per clutch we recorded was similar to that found by Bujes (1998), who worked in a similar geographic area with similar vegetation, soil, and climatic conditions to the Delta do Jacuí State Park.

Cabrera (1998) described the eggs of *P. hilarii* as spherical, with a white, smooth, and hard shell, and a diameter ranging from 27–37 mm. The recorded values in our study (from 30 to 37 mm) are within this range. The incubation period of the eggs of *P. hilarii* in natural conditions have been found to range from 70–140 days (Cabrera 1998). The longest incubation period recorded for this species, in artificial conditions, was 69 days, with a constant temperature of 34.5ºC (Piña and Argañaraz 2003). In our study, the incubation period was relatively long in both natural (157 to 271 days) and laboratory conditions (130 to 191 days), which probably was due to the smooth

temperatures in natural conditions and the absence of supplemental heating in the laboratory. Comparatively, eggs of *P. geoffroanus* incubated in mean temperatures between 27.3°C and 30.0°C, had incubation periods that varied from 115 to 186 days (Lisboa et al. 2004). Incubation periods for clutches exposed to varying thermal regimes ranged from 156 to 173 days (Kardon 1981), 206 and 319 days (Guix et al. 1989), and 149 and 331 days (Molina 1989).

Under natural conditions, incubation temperatures varied from 17 to 36ºC in the nest chamber, while at the soil surface they varied from 12 to 47ºC, and in the air ranged from 13 to 37ºC. In Australian chelonians, with nests at a depth of 180 mm, the temperature extremes in the interior of the egg chamber were 18 and 26ºC (Goode and Russell 1968). The location of the nest influenced the variation in temperature in the egg chamber. Nests exposed to the sun during the morning and afternoon experience thermal oscillations. In one nest from the second temperature profile, the eggs experienced an increase in temperature of 10ºC followed by a decrease in 11ºC in a 24-hour period: temperatures oscillated from 19ºC (at 0900) to 29ºC (at 1500), and then to 18ºC (at 0600). Another nest located in an area that was exposed to the sun from 1100 to 1700 only experienced small thermal variation (~ 4ºC) during the same time-period.

Hewavisenthi and Parmenter (2001) observed that hatchlings of *Natator depressus* from eggs incubated in the laboratory, with temperatures oscillating

TABLE 3. Mean (sample size), standard deviation, and range of carapace length (CL), carapace width (CW), shell height (SH), plastron length (PL), plastron width (PW), and mass (g) of the hatchlings of *Phrynops hilarii* born in natural and artificial conditions.
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	Natural conditions	Artificial conditions
CL	36.74 (31)	42.91 (25)
	1.472, 35.2–40.1	0.959, 41–45.8
CW	28.88 (31)	34.01 (25)
	0.739, 27.2–30.1	1.271, 31.3–36.4
SH	13.37 (31)	15.21 (25)
	0.476, 12.8–14.6	1.503, 10–17.2
PL	31.42 (31)	37.75 (25)
	0.551, 30.4–32.8	0.848, 36.7–40.6
PW	18.48 (31)	18.61 (25)
	0.612, 17.3–19.2	2.25194, 16.2–23.6
MASS	8.74 (31)	13.56 (25)
	0.404, 8.15–9.60	0.718, 12.5–15.4

between 26 and 29ºC, were significantly larger and had less energetic reserves than those incubated at 32ºC. Eggs of *P. hilarii* that we incubated in artificial conditions also produced larger hatchlings than those incubated under natural conditions. The mean temperature in artificial conditions of incubation (22.2°C) was lower than the lowest mean temperature found inside the incubation chamber (24.2°C). Therefore, we believe that the thermal environment in the natural nests influenced the morphological and physiological characteristics (i.e., apparent egg yolk or not) in the hatchlings, similar to those observed in artificial conditions.

Hatchling *Emydura macquarrii* and *Chelonia expansa* (in the family Chelidae) had significant responses to the amount of rain and to the associated temperature changes (Booth 1998). In both species, hatchlings emerged from the nest during or after intense rainfall. We did not find a relationship between the emergence of hatchlings of *P. hilarii* and the amount of rain or temperature change.

Hatchlings in four of six nests that we monitored emerged at dusk (from 1800–2000). In one nest, the hatchlings emerged from 2200–0600, avoiding the high temperatures of the day. Hatchlings emerged alone, quickly, and in a synchronized way, thus supporting the observations made by Congdon et al. (1983). After emergence, hatchlings moved fast on the surface, probably searching for water, as suggested by Plummer (2007) for hatchling *Apalone mutica*.

We found a lag period between hatching and emergence in one nest (68 days), which has also been reported for marine (Christens 1990; Godfrey and Mrosovsky 1997) and other freshwater chelonians (Bager and Fagundes 2007; Greaves and Litzgus 2007). This temporal discontinuity may be enough to change the shape of the carapace of the neonates of *P. hilarii* from oval to the flat shape we observed in the species. This lag time leads to the consumption of the residual reserve of the yolk sac (Nagle et al. 2003), and higher temperatures of the microhabitat may trigger emergence (Plummer 2007).

Hatchlings of *P. hilarii* we incubated in artificial conditions were larger than those incubated in natural conditions, thus supporting the hypothesis that cooler incubation temperature yields larger hatchlings. The body size of hatchlings is an important component of later success (Miller et al. 1987; Janzen 1993; Janzen et al. 2000). In general, larger hatchlings have advantages over smaller hatchlings in the form of increased competitive ability such as increased locomotor performance (Packard and Packard 1988). On the other hand, small hatchlings have larger energetic reserves, which will support them for long periods (Gutzke et al. 1987; Packard et al. 1987, 1988). Hewavisenthi and Parmenter (2001) observed that embryos in humid and cold environments developed slowly, taking up more nutrients from the yolk sac. This resulted in larger hatchlings with smaller energy reserve. The same was observed for

the hatchlings of *P. hilarii* that we incubated in artificial conditions, but the development of the hatchlings was faster. Conversely, the development of hatchlings from natural nests was slower and the animals took up more nutrients from their yolk sac, developing into smaller hatchlings with larger nutrient reserves.

Our results for *P. hilarii* provide valuable baseline data for this species. Incubation times suggest that they do not have direct development. Future research should address if embryonic diapause and/or embryonic aestivation are responsible for these long incubation times, both in nature and under laboratory conditions. The effect of varying hydric and temperature conditions in the laboratory should be undertaken to understand embryogenesis in this species and what mechanisms in nature control development in this species.

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