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Pivotal Temperature and Hatchling Sex Ratio of Olive Ridley Sea Turtles *Lepidochelys olivacea* from the South Atlantic Coast of Brazil

Vinicius D. Castheloge[1]***, Marcelo Renan de D. dos Santos***[1,2]***, Jaqueline C. de Castilhos***[3]***, Paulo Roberto de J. Filho***[2]***, Levy de C. Gomes1, Rute Beatriz G. Clemente-Carvalho***[4,6]***, and Paulo D. Ferreira***[1,5]

[1]*Universidade Vila Velha, Rua Comissário José Dantas de Melo, 21, Boa Vista, Vila Velha, 29102-770, Espírito Santo, Brazil*
[2]*Instituto Marcos Daniel, Rua Fortunato Ramos, 123, Santa Lucia, Vitória, 29055-290, Espírito Santo, Brazil*
[3]*Projeto Tamar/ICMBIO, Reserva Biológica de Santa Isabel, Pirambu, 49190-000, Sergipe, Brazil*
[4]*Department of Biology, Queen's University, Kingston, K7L 3N6, Ontario, Canada*
[5]*Departamento de Gemologia, Universidade Federal do Espírito Santo, Campus Goiabeiras, Vitória, 29075-910, Espírito Santo, Brazil*
[6]*Corresponding author, e-mail: rute_beatriz@hotmail.com*

***Abstract.*—Olive Ridley Sea Turtles (*Lepidochelys olivacea*) exhibit temperature-dependent sex determination (TSD). Knowledge of the influence of physical factors such as temperature on the nest environment is essential for understanding embryonic development and sex determination in sea turtles. Using field and laboratory data, we estimated the pivotal temperature, thermal limits, and partial sex ratios in Olive Ridley Sea Turtles. *Ex situ* analysis indicated that the pivotal temperature of *L. olivacea* in Sergipe, Brazil, is 30.7° ± 1.5° C, with estimated thermal limits for embryo viability ranging between 26° C and 34° C. The *in situ* sex ratio estimated for the last third of the nesting season (Pirambu Beach, Sergipe) was female-biased. Despite our results being derived from a fraction of the breeding season, this is a first important step for understanding hatching success and sex ratios *L. olivacea* in Brazil. Establishing the pivotal temperature and thermal limits from the whole season is undoubtedly important to generate more robust sex ratio estimates and to better characterize thermal limits for *L. olivacea*.**

Key Words.—incubation temperature; reptile; sea turtle; temperature-dependent sex determination; thermal limits

Introduction

The Olive Ridley Sea Turtle (*Lepidochelys olivacea*) has a circumtropical distribution with nesting sites occurring in nearly 60 countries (Marcovaldi and Marcovaldi 1999; Castilhos and Tiwari 2006; Silva et al. 2007). This species is considered Vulnerable by the International Union for Conservation of Nature (IUCN) due to a diverse array of threats (Abreu-Grobois and Plotkin 2008). Threats include incidental fishery juvenile bycatch (Castilhos et al. 2011), egg harvest (Cornelius et al. 2007), and shifts in beach characteristics, contributing indirectly to changes in turtle reproduction, and consequently to their population decline (Castilhos et al. 2011).

Lepidochelys olivacea exhibit temperature-dependent sex determination (TSD) in which higher temperatures produce females and cooler temperatures produce males (Bull et al. 1982; McCoy et al. 1983; Godfrey and Mrosovsky 2001; Godfrey et al. 2003; Mrosovsky et al. 2009). The projected increase in global temperatures raises critical concerns for *L. olivacea* and other sea turtle populations because changes in temperature might dramatically impact hatchling sex ratios and could also impact the development of the eggs by increasing mortality (Wood et al. 2014; Wyneken and Lolavar 2015) or by producing only females. The incubation temperature at which a 1:1 male to female sex ratio is produced, or pivotal temperature, for *L. olivacea* was previously determined for populations in Costa Rica (30–31° C; McCoy et al. 1983; Wibbels et al. 1998) and India (29.5° C; Godfrey and Mrosovsky 2006). Different studies have reported varying pivotal temperature for different turtle species and populations (Yntema and Mrosovsky 1982; Rimblot et al. 1985; Standora and Spotila 1985; Marcovaldi et al. 1997; Binckley et al. 1998).

Because *L. olivacea* is a widespread species, it may show adaptive responses to geographic variation in environmental factors. Determining this trait in nesting populations of Brazil is particularly important for the management and conservation of this species because nesting areas are highly affected by coastal development and transformation of natural conditions of the beaches

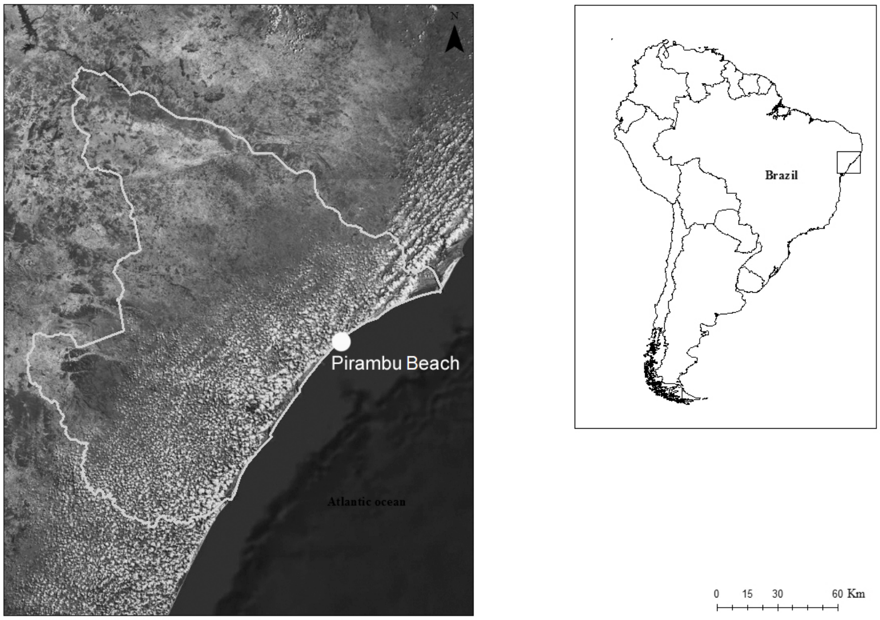

Figure 1. Location of the sampling site for nests of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*), Pirambu Beach, Sergipe State, northeastern Brazil. (Map created on ArcGIS 10, Esri, Redlands, California, USA).

(Castilhos et al. 2011). There were no historical data about the *L. olivacea* population in Brazil before the 1980s, and population demography has been studied based on number of nests in remnant areas (Castilhos et al. 2011). Currently, it is known that both the geographic range of the species and number of nesting sites of *L. olivacea* are small, with distribution occurring from the South of Alagoas state to the North of Bahia state and nesting occurring mainly in Sergipe state (da Silva et al. 2007; Castilhos et al. 2011).

Recently, Castilhos et al. (2011) following IUCN criteria, considered the conservation status for *L. olivacea* in Brazil as Endangered due its isolation from the other populations. Despite recent increases in nesting, these authors pointed out that this information must be viewed with caution, and long-term studies are required to accurately estimate population abundance and demography in Brazil. The objective of our study was to determine the pivotal temperature and estimate partial primary sex ratios for *L. olivacea* in the northeastern Brazil nesting site. To this end, we combined field and laboratory experiments to compare the sex ratios of hatchlings and pivotal temperature in the laboratory versus natural nests.

Materials and Methods

Laboratory pivotal temperatures.—We collected 150 *L. olivacea* eggs on 1 February 2013 from four nests at Pirambu Beach, in Sergipe, Northeastern Brazil (10°44’20.45”S, 36°50’56.68”W; Fig. 1) for determination of pivotal temperature. The beach is approximately 45 km in length, and it is comprised of open sand backed by low-lying dunes and salt marshes. Some sparse maritime vegetation is present far from the shore. We solely sampled the nests for which we found the female digging and laying eggs, and we collected all eggs < 6 h after females laid them. Each nest had approximately 100 eggs, and we avoided collecting all the eggs from any of the four sampled nests. We preferentially sampled the nests in a range of approximately 5 km in distance from the monitoring base to reduce variation caused by nest site microclimates. We placed the eggs from each nest into a labeled styrofoam box filled with sand and moistened vermiculite. This procedure ensured protection of the eggs against rotation and mechanical shock during transportation and enabled maintenance of a suitable temperature for the developing embryos. We transported the eggs to the Terrestrial and Aquatic Ecology Laboratory, Universidade Vila Velha (UVV), Espírito Santo, Brazil (approximately 7 h of traveling).

We distributed 150 eggs among 13 incubators with the same dimensions (350 × 440 × 390 mm). Temperatures in the incubators ranged between 26° C and 35° C (Table 1), with average temperature varying by ± 0.5° C within each incubator. We maintained incubator temperature using an incandescent light bulb (100 W) attached to a thermostat. We filled each incubator with moistened vermiculite and monitored temperatures of the incubator using iButton data loggers (Model DS1921G, Maxim Integrated, San Jose, California, USA). We monitored the incubators three to five times per day to check for signs of pipping or hatching, measure the temperature, and moisten the vermiculite. All procedures were completed swiftly so as not to disturb the interior temperature of the incubators. We used two data loggers in each incubator, one placed in the middle of the eggs and the other on the top of the clutch to monitor the temperatures throughout incubation. We considered only the dataset recorded on the data logger that was placed in the middle of the chamber to calculate the average incubation temperature.

We set up the iButtons to record temperature every 15 min, totaling 96 measurements per day. The accuracy of the loggers was ± 1.0° C and the resolution was 0.5° C, which resulted in a ±1.5° C range associated with each reported temperature. All the data loggers were calibrated by the company Metrology (Vitória, Espírito Santo, Brazil) before the pivotal experiment. We assigned emergence date as the day when we detected disturbances to the vermiculite surface either caused by movement of the hatchlings underneath or emerged hatchlings.

After emergence, we transferred the hatchlings to the sea turtle center in Vitoria, Espírito Santo (ES), which is part of the Projeto Tartaruga Marinha/Instituto Chico Mendes de Conservação da Biodiversidade (TAMAR/ICMBio), the government organization responsible for protecting marine turtles in Brazil. At the TAMAR base, all work was done by government employees. All hatchlings produced in the incubation experiment were maintained in one 1,000-L plastic tank with saltwater at controlled temperatures between 27° C and 28° C and were fed daily with shrimp. Captive-rearing permitted gonadal development, improving the sex identification of the individuals. After a period of two to four months, we euthanized individuals via an intracardiac injection of 0.5 ml of sodium thiopental 25%, excised the gonads for histological examination, and stained tissues with hematoxylin-eosin. We classified the gonads as either ovaries or testes according to the criteria of Yntema and Mrosovsky (1980). We used the proportions of males and females from each incubator to calculate the pivotal temperature using the TSD software V 4.0.3 (Godfrey et al. 2003), applying Hill equation and TSD IA pattern.

Study area, nest monitoring, and data analysis.— In collaboration with TAMAR/ICMBio, we collected the data of the nests for which we witnessed the female laying eggs. We monitored 55 *L. olivacea* nests from egg-laying to emergence during January to March 2013 on the coast of Pirambu Beach of Sergipe State, Brazil (Fig. 1). We recorded the temperatures at 60-min intervals using calibrated data loggers (Model DS1921G) throughout the incubation period. We placed data loggers in the middle of each clutch the same day of the oviposition by carefully removing the sand and some eggs. The first measurements occurred less than 24 h after oviposition. We monitored the nests daily during the entire period of incubation to check for signs of egg hatching and hatchling emergences.

We estimated the laboratory pivotal temperature and the relationship between the incubation temperature and sex ratios of the laboratory hatchlings. Based on these temperatures, we were able to calculate the sex ratio of the *in situ* nests. We used two methods to calculate *in situ* sex ratios; first, we used temperatures of the middle third of the incubation period (Godfrey et al. 2003), and second, we used the constant temperature equivalent method (CTE; Georges et al. 1994). The CTE describes the contribution of certain incubation temperature ranges in relation to the entire incubation period. We performed both analyses using the TSD software V 4.0.3 (Godfrey et al. 2003). Data did not meet the assumptions for a parametric test, so we used the non-parametric Kruskal-Wallis test to identify possible differences in the middle third average temperatures and the CTE between the sampled sections of the beach. Because conditions along the length of the beach were similar (e.g., sand type, beach profile, albedo, and nearby vegetation), we monitored only the first 6 km of the beach as a representative sample. To determine whether differences between temperatures of the middle third of incubation and the CTE were significant, we used the Mann-Whitney U-test (the data was not normal and homoscedatic). We evaluated the influence of the middle third incubation temperature on hatchling sex ratios using Hill's power equation estimated on TSD software. We used Systat 12 software (Wilkinson 2012) for testing with $\alpha = 0.05$.

Results

Laboratory pivotal temperatures.—Of the 150 eggs used in the experiment, only 33% (50 eggs) hatched in the laboratory. The pivotal temperature estimated for *L. olivacea* in Pirambu using histological analysis of gonads was 30.7 ± 1.5° C. The transitional range around the pivotal temperature estimated by TSD software (temperature range from 5–95% female sex ratio) extended from 29.6° C to 31.4° C (Table 1, Fig. 2). Male and female hatchlings occurred in different proportions (Table 1). For all incubators with an average iButton temperature of 29.9 ± 0.5° C or below, all males were obtained except for one female. For all incubators with an average temperature of 31.2 ± 1.9° C or above, only females were produced. Our data suggest that the thermal limits for embryonic development ranged between 26° C and 34° C (Table 1, Fig. 2) because these extreme temperatures produced either two or no live hatchlings (Table 1). Of the 13 incubators, we only considered 12 for the analysis because one incubator produced no viable hatchings. The Hill equation curve explained the

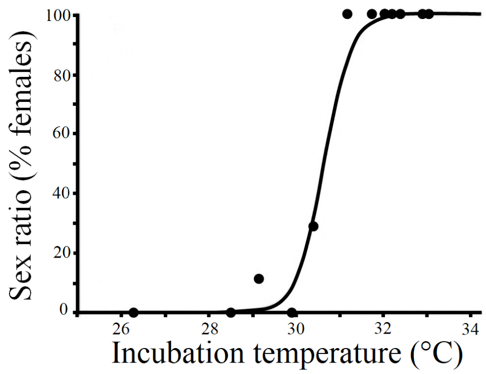

Figure 2. Curve estimated in TSD software applying Hill's power equation ($r^2 = 0.83$, $P = 0.92$, AIC = 9.797). The curve represents the relationship between temperature in the middle third of the incubation period and sex ratios for nests of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*) sampled from Pirambu Beach, Sergipe State, northeastern Brazil, and incubated *ex situ*.

relationship from 12 incubators to calculate the CTE (Georges et al. 1994): Length of Incubation (days) = 38,439 × (Constant Temperature ° C)$^{-1.936}$.

Nest beach analysis.—Clutches monitored *in situ* (n = 55) were laid during the last third of the nesting season, between 7 January and 5 March 2013. The clutches emerged between 21 February and 1 May 2013 and had an average incubation duration of 49.5 d (SD = 3.1; Appendix). The mean middle third temperatures for individual nests ranged from 28.0° C to 32.8° C, as recorded by the iButtons. The estimated hatchling sex ratio of Pirambu nests was female-biased for the mean middle third temperature (95% females) and for CTE (98% female; Table 2). The sex ratio did not differ among the sampled sections of beach for either middle third incubation periods ($\chi^2 = 0.747$, df = 3, $P = 0.862$) or CTE ($\chi^2 = 1.001$, df = 3, $P = 0.801$); however, there was a significant difference between the hatchling sex ratio calculated from mean mid-third temperatures and those calculated using CTE (U = 2799, df = 1, $P < 0.001$). Of the 55 monitored nests, only one was predicted to consist exclusively of male hatchlings. The mean temperature calculated for this nest during the entire period of incubation and middle third period was 27.6° C, and 28.0° C, respectively.

Table 2. Sex ratios (average proportion of females ± 95% confidence interval) and the number of nests of Olive Ridley Sea Turtles (*Lepidochelys olivacea*) sampled (n) along 4 km of beach as estimated using iButton-recorded temperatures from the middle third of the incubation period and using the constant temperature equivalent (CTE).

		Sex Ratio	
Km	Nests (n)	Middle Third	CTE
1	2	0.956 ± 0.059	0.997 ± 0.004
2	16	0.970 ± 0.037	0.999 ± 0.005
3	21	0.963 ± 0.055	0.998 ± 0.008
4	16	0.911 ± 0.245	0.936 ± 0.250
Total	55	0.950 ± 0.027	0.982 ± 0.031

Table 1. Average temperature (AT) ± 95% confidence interval, number of eggs incubated (n), proportion of eggs hatched (PEH), number of males produced (Males), number of females produced (females), and hatchling sex ratios (HSR) in proportion of females of Olive Ridley Sea Turtles (*Lepidochelys olivacea*) produced under different laboratory incubation temperatures.

AT (° C)	n	PEH	Males	Females	HSR
26.3 ± 0.3	10	0.200	2	0	0.000
28.5 ± 0.2	12	0.250	3	0	0.000
29.2 ± 0.2	12	0.750	8	1	0.111
29.9 ± 0.5	12	0.583	7	0	0.000
30.4 ± 0.2	12	0.583	5	2	0.286
31.2 ± 1.9	12	0.083	0	1	1.000
31.8 ± 1.2	12	0.250	0	3	1.000
32.1 ± 0.4	12	0.500	0	6	1.000
32.2 ± 0.4	12	0.167	0	2	1.000
32.4 ± 1.9	12	0.083	0	1	1.000
32.9 ± 0.6	12	0.417	0	5	1.000
33.1 ± 0.3	10	0.400	0	4	1.000
34.4 ± 0.9	10	0.000	0	0	---

Discussion

In this study, the pivotal temperature determined for *L. olivacea* in Brazil was 30.7 ± 1.5° C, similar to conspecific populations in Costa Rica (30–31° C; McCoy et al. 1983; Wibbels et al. 1998) and India (29.5° C; Godfrey and Mrosovsky 2006). Despite the wide geographical distribution, sea turtles have similar pivotal temperatures among different species and populations (Mrosovsky 1994; Wibbels et al. 1998; Godley et al. 2002), indicating that it can be a heritable trait (Refsnider and Janzen 2016). Therefore, latitudinal variations among nesting rookeries and their associated differences in climate conditions do not result in changes in the pivotal incubation temperatures (Mrosovsky 1988).

Our *ex situ* analysis indicated that the pivotal temperature in *L. olivacea* is slightly higher than other sea turtle species, including *Caretta caretta* (29.1° C; Marcovaldi et al. 1997), *Eretmochelys imbricata* (29.6° C; Godfrey et al. 1999), *Chelonia mydas* (28.5–30° C; Standora and Spotila 1985), and *Dermochelys coriacea* (29.4–29.7° C; Rimblot et al. 1985; Binckley et al. 1998). This higher pivotal temperature compared to those of other sea turtle species along Brazilian coast, is likely why *L. olivacea* has a limited nesting range of approximately 200 km from south Alagoas to north of

Bahia in Brazil. Few nesting females have been found south of Bahia, Espírito Santo and Rio de Janeiro states, and they can be considered as infrequent nesting sites of *L. olivacea* (da Silva et al. 2007). Therefore, thereby concentrating the largest number of nesting females along the Sergipe coast makes this region an important nesting area in Brazil for this species.

Our *in situ* data suggest that the average *L. olivacea* nest temperatures observed during the final third of the nesting season were far from the thermal limits and did not hamper embryonic development. In addition, our *ex situ* data suggested that the thermal limits for embryonic development ranged approximately between 26° C and 34° C; however, we were unable to calculate the hatching success of the nesting beach because we did not study all of the nests during the whole season. In addition, our data do not reflect the sex ratios of all nests laid in Sergipe because we sampled only the last third of nesting season. Previous studies reported that the hatchling sex ratios may vary between seasons (Hernández-Echeagaray et al. 2012) and nests (Booth and Astill, 2001). Moreover, poorly managed hatcheries may provide unnatural temperature variation inside the incubators (Maulany et al. 2012). On the other hand, positive results were reported when techniques of beach management (including nest relocation to hatcheries) were well applied, and hatching success and sex ratios were not significantly different between *in situ* and relocated nests (García et al. 2003).

The estimated sex ratios of *L. olivacea* did not vary along the sampling area (5 km), which indicates that the characteristics of the beach along its length may be similar. Despite the statistical difference between the sex ratios obtained from the middle third of the incubation period (Godfrey et al. 2003) and the constant temperature equivalent methods (CTE), we assume that there is no biological difference that may imply variation in the population structure. Both methods indicated that the populations structure has a female-bias tendency.

Our study represents a first step that contributes to the conservation of *L. olivacea* in the South Atlantic by providing new information about pivotal temperature and hatchling sex ratios. These findings will be valuable for management programs that aim to conserve populations of turtles, potentially mitigating circumstances that can skew the *in situ* sex ratio. Estimates of hatchling sex ratios and pivotal temperatures of all *L. olivacea* nesting beaches and for the whole breeding season in Sergipe would provide more robust information across spatial and temporal scales. These data are needed to provide insights into the potential for climate change to affect hatchling sex ratios and possible phenotypic plasticity of nesting females to respond to these changes.

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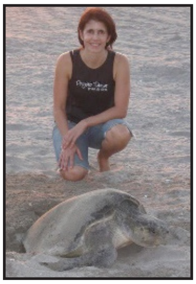
Vinicius Davel Castheloge received his M.S. in Ecology of Ecosystems from the Universidade Vila Velha, Espírito Santo, Brazil (2011). Vinicius is currently working on his Ph.D. at the same University, developing a study that involves records of marine animal strandings on the Brazilian coast. (Photographed by Lillian Jardim Guimarães).

Marcelo Renan de Deus dos Santos is a Veterinarian who graduated from the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (1995). He received his M.S. in Animal Biology from the Universidade Federal do Espírito Santo, Brazil (2005), and Ph.D. in Ecology of Ecosystems from the Universidade Vila Velha, Espírito Santo, Brazil (2015). Marcelo is the President of the Instituto Marcos Daniel and Professor at the Universidade Vila Velha, dedicating his work on Conservation of Marine Turtles and Caimans. (Photographed by Maurizélia Brito).



Jaqueline C. De Castilhos received her B.S. degree in Biology from the Universidade Federal de Sergipe, Brazils, and M.S. degree from the Universidade Federal da Bahia, Salvador, Brazil. Jaqueline is currently the Technical Coordinator of TAMAR Program (Projeto Tartaruga-Marinha) in Sergipe. (Photographed by Perla Torres).

Paulo Roberto de J. Filho graduated in Biology from the Universidade Vila Velha, Espírito Santo, Brazil. He has been working as a Biologist Technician mainly on herpetofuna at the company CTA-Serviços em Meio Ambiente. (Photographed by Paulo R. de J. Filho).

Levy de Carvalho Gomes received his M.S. degree from the Universidade Federal de Santa Maria Brazil, and Ph.D. degree from the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA). His research interests are focused on ecotoxicology and how environmental changes have impacted aquatic organisms such as fishes and marine turtles. He is currently a Professor at the Universidade Vila Velha, Espírito Santo, Brazil. (Photographed by Adriana Chippari-Gomes).

Rute B. G. Clemente-Carvalho is a Research Associate at Queen's University, Kingston, Ontario, Canada. She completed her M.Sc. at the Museu de Zoologia da Universidade de São Paulo, Brazil (MZUSP), and Ph.D. at Universidade Estadual de Campinas, Brazil (UNICAMP). Her research interests center on population genetics and systematics, particularly on patterns of morphological differentiation and molecular divergence among populations across their geographic ranges. (Photographed by Haley Richardson).

Paulo D. Ferreira is a Geological Engineer and has a Ph.D. degree in Natural Sciences from Universidade Federal de Ouro Preto, Minas Gerais, Brazil. He has experience in Geosciences with research interests in how nest characteristics influence turtle reproduction. Paulo is a Professor at the Departamento de Gemologia at the Universidade Federal do Espírito Santo, Brazil. (Photographed by Vinicius D. Castheloge).

Appendix Table. Dates when eggs were laid and hatchlings emerged, and respective mean middle third temperatures and sex ratios (proportion female) for *in situ* nests of Olive Ridley Sea Turtles (*Lepidochelys olivacea*) monitored during the last third of the 2013 breeding season from Pirambu Beach, Sergipe State, northeastern Brazil.

Nest ID	Date Laid	Date Emerged	Mean Middle Third Temperature	Sex Ratio (Proportion Female)
507	7 January 2013	23 February 2013	31.5	0.926
509	7 January 2013	23 February 2013	31.9	0.977
510	7 January 2013	21 February 2013	31.2	0.848
516	8 January 2013	23 February 2013	31.3	0.884
534	7 January 2013	23 February 2013	31.4	0.902
535	7 January 2013	24 February 2013	31.6	0.946
536	8 January 2013	24 February 2013	31.5	0.928
543	11 January 2013	25 February 2013	32.2	0.991
545	8 January 2013	25 February 2013	31.6	0.948
546	8 January 2013	25 February 2013	31.9	0.980
554	8 January 2013	27 February 2013	32.4	0.995
556	8 January 2013	25 February 2013	31.8	0.975
573	9 January 2013	27 February 2013	32.1	0.987
574	9 January 2013	28 February 2013	32.6	0.998
575	9 January 2013	1 March 2013	32.5	0.997
582	11 January 2013	1 March 2013	31.3	0.878
583	10 January 2013	1 March 2013	32.1	0.990
586	14 January 2013	1 March 2013	31.4	0.915
587	14 January 2013	1 March 2013	32.8	0.998
588	14 January 2013	2 March 2013	31.4	0.907
589	11 January 2013	2 March 2013	31.9	0.981
590	11 January 2013	1 March 2013	32.3	0.994
595	14 January 2013	5 March 2013	32.6	0.998
612	21 January 2013	5 March 2013	31.9	0.982
614	16 January 2013	5 March 2013	32.1	0.809
615	16 January 2013	6 March 2013	32.4	0.996
617	16 January 2013	5 March 2013	32.0	0.983
628	18 January 2013	8 March 2013	32.3	0.994
629	18 January 2013	5 March 2013	32.6	0.998
641	31 January 2013	13 March 2013	32.0	0.987
647	20 January 2013	13 March 2013	28.0	0.000
659	26 January 2013	15 March 2013	32.0	0.983
660	26 January 2013	15 March 2013	31.2	0.867
661	26 January 2013	16 March 2013	31.9	0.978
663	26 January 2013	16 March 2013	31.9	0.982
673	29 January 2013	21 March 2013	31.9	0.976
762	22 February 2013	11 April 2013	32.0	0.987
763	15 February 2013	11 April 2013	32.3	0.995
764	22 February 2013	11 April 2013	31.8	0.971
778	22 February 2013	11 April 2013	32.7	0.998
779	22 February 2013	11 April 2013	32.5	0.997
782	23 February 2013	11 April 2013	31.5	0.943

Appendix Table (continued). Dates when eggs were laid and hatchlings emerged, and respective mean middle third temperatures and sex ratios (proportion female) for *in situ* nests of Olive Ridley Sea Turtles (*Lepidochelys olivacea*) monitored during the last third of the 2013 breeding season from Pirambu Beach, Sergipe State, northeastern Brazil.

Nest ID	Date Laid	Date Emerged	Mean Middle Third Temperature	Sex Ratio (Proportion Female)
785	23 February 2013	11 April 2013	32.8	0.999
787	23 February 2013	11 April 2013	32.3	0.994
788	22 February 2013	16 April 2013	31.8	0.971
808	22 February 2013	11 April 2013	32.4	0.996
809	23 February 2013	11 April 2013	32.6	0.998
811	23 February 2013	11 April 2013	32.4	0.995
812	23 February 2013	11 April 2013	32.7	0.998
813	22 February 2013	11 April 2013	32.7	0.998
815	22 February 2013	11 April 2013	32.8	0.999
884	2 March 2013	22 April 2013	32.1	0.988
893	5 March 2013	1 May 2013	32.0	0.983
894	5 March 2013	1 May 2013	31.9	0.980
895	4 March 2013	1 May 2013	31.8	0.969