

Temperature-Dependent Sex Determination in the Diamond-backed Terrapin (*Malaclemys terrapin*)

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ABSTRACT.—Most Emydidae, including Diamond-backed Terrapins (*Malaclemys terrapin*), have temperature-dependent sex determination. However, the full relationship between incubation temperature and offspring sex in the Diamond-backed Terrapin has not been reported, and the pivotal temperature, range of transitional temperatures, and the temperature-sensitive period have not been investigated fully. Here we report on these parameters, comparing our data with data from other emydid turtles, and also comparing our laboratory data to field data collected previously for this population.

RESUMEN.—La mayoría de Emydidae, incluyendo a las Diamond-backed Terrapins (*Malaclemys terrapin*), tienen determinación de sexo por temperatura. Sin embargo, la relación entre temperatura de incubación y sexo de crías en el Diamond-backed Terrapin no ha sido reportada, ni el umbral de temperatura ni el período sensible a la temperatura han sido investigados. Aquí reportamos ambos fenómenos y los comparamos con otras Emydidae, así como con datos de campo de ésta población previamente colectados.

In their landmark paper, Ewert and Nelson (1991) reported sex determination data for a wide range of turtle species, laying the groundwork for multispecies analyses of sex determination patterns in reptiles. Of particular interest was the Emydidae, wherein both temperature-dependent sex determination (TSD) and genotypic sex determination (GSD). Among TSD turtles, there are two patterns known, TSD Ia and TSD II, but their evolutionary and ecological significance remains enigmatic (Ewert et al., 2004).

Although Ewert and Nelson (1991) reported detailed information for many emydids, their data for Diamond-backed Terrapins (*Malaclemys terrapin*) were limited. Their article showed only that Diamond-backed Terrapins have TSD, with all female hatchlings from eggs (Accomac County, Virginia; Hotaling, pers. comm.) incubated at 30°C and all male hatchlings from eggs (from Bergen County, New Jersey, E. Hotaling, pers. comm.) incubated at 24°C. Later, Jeyasuria et al. (1994) incubated Diamond-backed Terrapin eggs from St. Mary's County, Maryland, and found that high temperatures (31 and 32°C) resulted in 100% female hatchlings and low incubation temperatures (26 and 27°C) resulted in 100% male hatchlings and that eggs incubated at 29.5°C resulted in 13% male hatchlings. Thus, Diamond-backed Terrapins seem to be a typical TSD Ia species (Ewert and Nelson, 1991), with a single transitional temperature range; incubation temperatures below this range result in males, whereas incubation temperatures above this range produce females. The TSD I pattern is evident in most but not all emydid turtles. However, without sex ratio data from a wide range of temperatures, it is possible that a second transitional range exists, as is seen in TSD II species (Ewert and Nelson, 1991).

Pivotal (=threshold; Bull et al., 1982) temperature is the constant incubation temperature that results in 1:1 offspring sex ratios in TSD species (Mrosovsky and Pieau, 1991). This value has become an important component of models that relate incubation temperatures to offspring sex ratios (e.g., Georges et al., 1994; Doody et al., 2006) and is a common repeatable metric for intraspecific (e.g., Bull et al., 1982; Ewert et al., 2005) and interspecific (Sarre et al., 2004) comparisons. Less frequently calculated, yet perhaps as important for understanding TSD

(e.g., Hulin et al., 2009), is the transitional range of temperature (TRT). The TRT is the range of constant incubation temperatures that produce both sexes and may be associated with the likelihood of evolutionary response to selective pressures because of changing thermal environments (Hulin et al., 2009). Girondot (1999) pointed out that calculation of TRT depends on statistical technique, number of incubation temperatures, and samples sizes used.

There is significant genetic structuring across the Diamond-backed Terrapin range (Hart, 2005; Hauswaldt and Glen, 2005) that spans 2,000 km north–south and could include substantial differences in incubation conditions. Thus, different populations may have evolved different pivotal temperatures (Bull et al., 1982; Ewert et al., 1994, 2005) and different TRTs. Unfortunately, Jeyasuria et al. (1994) did not report obtaining males and females at more than a single incubation temperature, so their data cannot be used to estimate pivotal temperature or TRT (Girondot, 1999).

The temperature-sensitive period (TSP) (Bull and Vogt, 1981) is that portion of the incubation period during which incubation temperature can affect the eventual sex of hatchlings. Identifying the TSD for a species is critical for models that are used to explore the relationship between temperatures in natural nests and the sex of resulting offspring (e.g., Georges et al., 2004). The TSP has not been reported for Diamond-backed Terrapins, although Jeyasuria et al. (1994) and Jeyasuria and Place (1997) estimated the TSP for Diamond-backed Terrapins to start at embryonic stage 12–14, both without explanation. The TSP has been reported for four other emydid turtles: European Pond Turtle (*Emys orbicularis*) (stages 16–21; Pieau and Dorizzi, 1981), Red-eared Slider (*Trachemys scripta scripta*) (stages 16–19 or 20; Wibbels et al., 1991), Ouachita Map Turtle (*Graptemys ouachitensis*) (stages 16–22; Bull and Vogt, 1981); and Painted Turtle (*Chrysemys picta*) (stages 16–22; Bull and Vogt, 1981). All five of these studies used Yntema (1968) to identify embryonic stages, so their results are comparable.

Our goals were to 1) determine whether the Diamond-backed Terrapin is a TSD type Ia species as is common in emydid turtles, 2) calculate the pivotal temperature of sex determination and TRT for the northern Diamond-backed Terrapin, 3) explore geographic variation in pivotal temperature of sex determination and TRT in Diamond-backed Terrapins, and 4) test the hypothesis of Jeyasuria et al. (1994) regarding the TSP and

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DOI: 10.1670/13-188

developmental stage for Diamond-backed Terrapins. We explored these phenomena in Diamond-backed Terrapins because of our investigations into the relationship between temperatures in wild nests and offspring sex ratios (e.g., Scholz, 2007) in this species. We used constant temperature incubation temperatures to address goals 1–3 and shift-temperature experiments to address goal 4.

MATERIALS AND METHODS

We collected Diamond-backed Terrapins' freshly laid eggs from nests in 2009 and 2010 on Rulers Bar Hassock, a 463-ha island (40°57'N, 73°50'W) in the mainly estuarine Jamaica Bay Wildlife Refuge. This site is located in Kings and Queens counties, New York, and near the center of the range of the Northern Diamond-backed Terrapin subspecies *Malaclemys terrapin terrapin*. In both years, we transported eggs to the laboratory; there they were marked and mass was determined. Clutches were divided so as to maximize representation of clutches among treatments. They were buried in vermiculite that had been mixed 2 parts distilled water:1 part vermiculite by weight and placed in constant temperature incubators within 48 h of oviposition. The mass of eggs and containers was determined weekly during incubation, and evaporated water was replaced.

In 2009, we incubated eggs at constant temperatures (24, 25, 26, 27, 28, 29, 30, 31, and 32°C) until hatching. We added data for eggs incubated at constant 34°C from Giambanco (2003) where applicable; Giambanco collected eggs in 2000 from the same population and used the same methodology described here for constant-temperature experiments. After pipping, eggs were removed from the incubators and hatchlings were allowed to emerge fully from the eggs. Hatchlings were then euthanized via chemical injection 3–4 d postemergence. Sex was identified by gross inspection of gonads using the presence or absence of Müllerian ducts (indicating female and male, respectively), and both microscopic and macroscopic examinations of the external appearance of the gonads.

In 2010, we collected eggs and incubated them as described above, except we conducted shift experiments (Valenzuela, 2001) to identify the TSP. We used baseline temperatures of 25 and 31°C. Eggs were held at one of the single baseline temperatures through most of incubation, but they were shifted to a different temperature for a specific length of time (Figs. 1 and 2). The baseline temperatures for the shift experiments (25 and 31°C) were chosen as likely to be male producing and female producing, respectively, based on results from 2009 experiments. We predicted incubation duration for the shift experiments based on results from 2009 experiments, and we divided the predicted incubation duration for the 31°C baseline into 5-d intervals and the predicted incubation duration for the 25°C baseline into 7-d intervals (Figs. 1 and 2). At each time interval when eggs were moved from their assigned baseline incubation temperature to the shift temperature, five eggs were chosen randomly for preservation. With these eggs, we used a bright light to locate an area within the shells without blood vessels; there, we used forceps to peel back the eggshells and expose the embryos. The embryos were removed and placed in Bouin's solution for preservation. Preserved embryos were staged according to Yntema (1968) and Greenbaum (2002).

We analyzed the constant temperature and sex of offspring data (including that of Giambanco [2003]) to assess sex determination mode and estimate both TSP and TRT with the

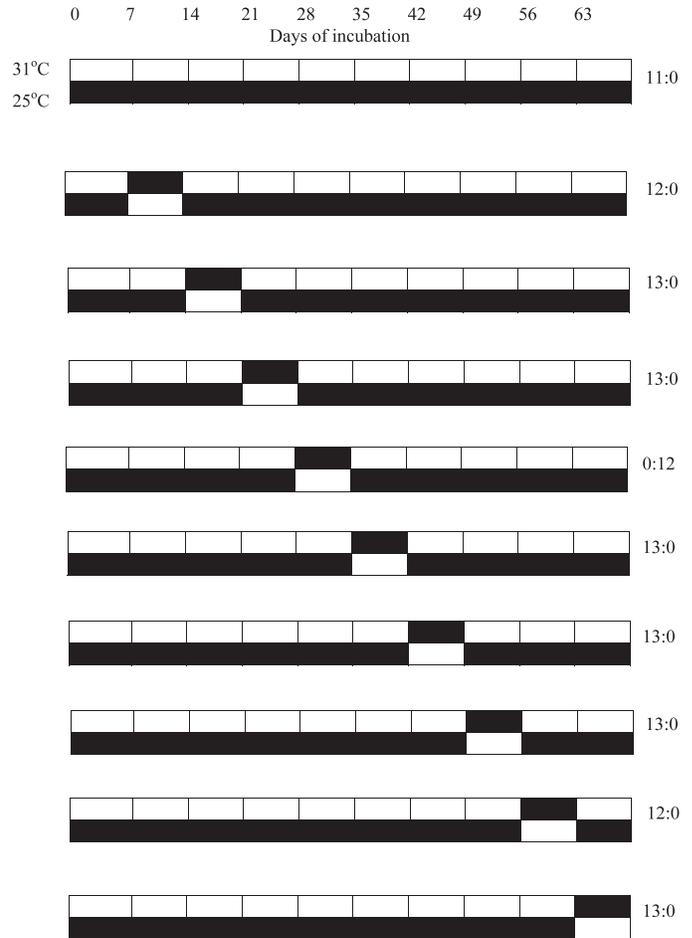


FIG. 1. Shift experiments with 25°C baselines, indicating shift periods and resulting sex ratios (male:female).

data from Diamond-back Terrapins from New York and Maryland (Jeyasuria et al., 1994) using the program TSD 4.0.3 (Girondot, 1999, 2012; Godfrey et al., 2003). Sex ratio data were fitted using several models (GSD, logistic, Hill, and Richards) using maximum likelihood with binomial distributions. The GSD model for genotypic sex determination is a model in which sex ratio has a predicted value of 0.5 and sex ratio is independent of incubation temperature. The logistic and Hill models both use a sigmoidal equation with two parameters, whereas the Richards model uses a sigmoidal equation with three parameters. Goodness of fit testing was based on likelihood ratios between the saturated model ($df_1 = 0$) and fitted model ($df_2 = [\text{number of temperatures}] - [\text{number of estimated parameters}]$). Comparisons between different models were conducted using Akaike Information Criterion (AIC), a method to rank different models by order of merit by penalizing for too many parameters. Akaike weight is a method that converts AIC into probabilities assessing whether each model is really the best choice among the tested models (Burnham and Anderson, 2013).

RESULTS

The GSD model can be excluded from fitting the New York data on the basis of a very high AIC ranking and very low weight (goodness of fit < 0.001, Table 1). The logistic and Hill

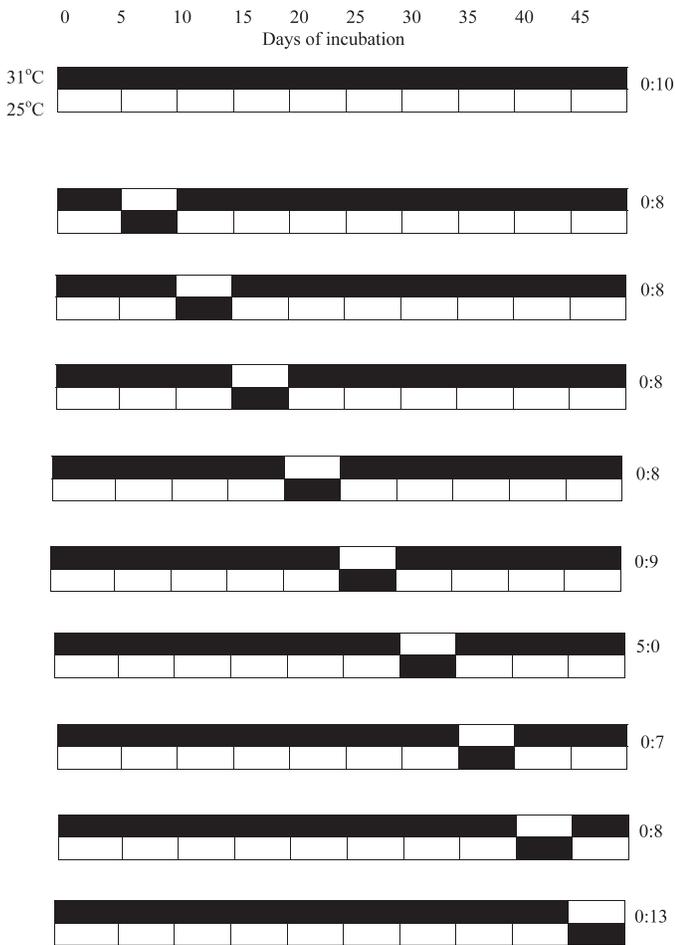
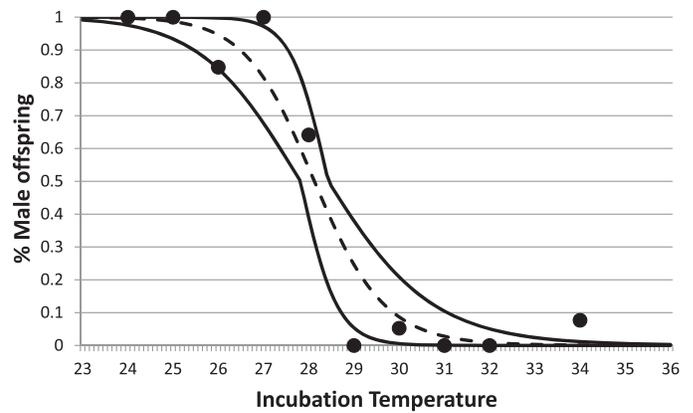


FIG. 2. Shift experiments with 31°C baselines, indicating shift periods and resulting sex ratios (male:female).

models both performed better than the Richards model, based on AIC ranking and Akaike weight. However, although the logistic and Hill models cannot be distinguished, we used the logistic based on parsimony because the Hill model is a special case of the logistic model with the x-axis being log transformed.

Goodness of fit testing for New York data rejected the model as fitting the data correctly ($p = 10^{-5}$). Observations at four incubation temperatures were particularly unlikely, such as the male observed at 34°C. However, even if this observation is removed from the data set, the model is still rejected ($p = 2.10^{-3}$), because there are significant variations in sex ratio among incubation temperatures that are not well represented by this model (Fig. 3). This could be due to fluctuation or



Sample sizes: 21 29 46 19 53 12 38 42 29 13

FIG. 3. Relationship between constant incubation temperature and offspring sex (percentage of males) in the Diamond-backed Terrapin. Plotted points indicate actual data; curves indicate the Hill equation (dashed line) fit to data bracketed by confidence interval of 2 SDs (solid lines).

nonstability of temperature within incubators or maternal effects.

The pivotal temperature for the New York data was 28.16°C and the TRT was 25.88–30.45°C (Fig. 3). Incubation duration at constant 25°C averaged 69.4 d (SD = 2.0); incubation duration at constant 31°C averaged 43.2 d (SD = 1.3).

Only two incubation temperatures are available for the New Jersey and Virginia populations, and none of these temperatures produced mixed sex ratios. With such a situation, it was impossible to fit these data to any model, and it did not make sense to add them to our other data sets.

The GSD model can be excluded from fitting the Maryland data on the basis of a very high AIC ranking and very low Akaike weight (goodness of fit < 0.001; Table 1). The logistic model (goodness of fit = 1.00) performed better than the Hill or Richards models, based on AIC ranking and Akaike weight. The pivotal temperature for the Maryland data was 29.25°C and the TRT was 28.85–29.64°C.

The combined data set (New York data and Maryland data) had a high AIC value and low Akaike weight (Table 1), especially in comparison with the two data sets analyzed separately (Table 1). This indicates significant differences between the data sets (goodness of fit < 0.001).

Our shift experiment with the 25°C baseline indicated that the TSP occurred between incubation days 28 and 35, 40.3–50.4% of incubation (Fig. 1). All five embryos preserved from 25°C baseline after 28 days of incubation were at developmental stage 16 as described in Yntema (1968) and Greenbaum (2002).

TABLE 1. Comparison of model fits among the populations of *M. terrapin* using AIC and Akaike weight.

Population	Model	AIC	Akaike weight
New York	GSD	300.01	2.785×10^{-55}
New York	Logistic	50.52	4.165×10^{-1}
New York	Hill	50.46	4.303×10^{-1}
New York	Richards	2.07	1.532×10^{-1}
Maryland	GSD	176.141	8.44×10^{-38}
Maryland	Logistic	7.129	4.237×10^{-1}
Maryland	Hill	7.14	4.20×10^{-1}
Maryland	Richards	9.13	1.56×10^{-1}
New York and Maryland combined	Logistic	69.18	3.13×10^{-3}
New York and Maryland separate	Logistic	57.65	9.97×10^{-1}

The shift experiment with the 31°C baseline indicated that the TSP occurred between incubation days 30 and 35, 69.4–81.0% of incubation (Fig. 2). Four of the five embryos preserved from 31°C baseline after 30 d of incubation were malformed, and we could not stage them reliably. The remaining embryo was at developmental stage 23. The embryos from the previous shift (removed at day 25) were at developmental stage 22; the embryos from the subsequent shift (removed at day 35) were at developmental stage 24.

DISCUSSION

We found that Diamond-backed Terrapins have TSD Ia pattern, as originally suggested, based on limited data, by Ewert and Nelson (1991). The pivotal temperature for the New York population is 1.09°C cooler than Diamond-backed Terrapins from populations from Maryland. These results span the two most northern of the six genetically based management units delineated by Hart (2005) and a range of ~400 km, which is only ~20% of the Diamond-backed Terrapin north-south distribution. Because of the large fraction of the Diamond-back Terrapin range for which TSD data are unknown, there is still considerable possibility for even more variation in pivotal temperature and TRT in this species, as has been demonstrated in other turtles (Bull et al., 1982; Ewert et al., 1994, 2004, 2005). However, our results indicate that pivotal temperatures and TRT for both populations of Diamond-backed Terrapins are within the range of other emydid turtles (28–29°C and 1–2°C, respectively, e.g., Pieau, 1976; Bull et al., 1982; Crews et al., 1991; Ewert and Nelson, 1991; Ewert et al., 2004; Hulin et al., 2009).

Our finding that the TSP for Diamond-backed Terrapins corresponds with embryonic stages 16–23 is not surprising because it is consistent with other emydid turtles (Bull and Vogt, 1981; Pieau and Dorizzi, 1981; Wibbels et al., 1991). Mrosovsky and Pieau (1991) suggested that the TSP in turtles occurred in the middle third of incubation. Although our findings confirm this for eggs incubated at the 25°C baseline, the TSP occurred in the last third of incubation for eggs incubated at 31°C. The cause of this striking variation is unclear, and we are unaware of similar results in any other species. In all cases, the same guide to staging embryos (Yntema, 1968) was used.

Our results have potentially valuable applications. For example, Scholz (2007) recorded temperatures in 82 Diamond-backed Terrapin nests throughout incubation at our study site in 2004. Average temperatures for these nests varied considerably (18.9–27.0°C, mean = 25.2°C, SD = 1.6). Therefore, these nests apparently spent relatively little time near or above the pivotal temperature and were probably biased strongly toward male hatchlings. Further analysis is needed to investigate the relationships between constant temperature incubation results reported here and variable temperature incubation patterns observed in the field.

Acknowledgments.—We are grateful to B. Clendenning, J. Joseph, and C. Peterson for assistance with techniques and data collection. E. Hotaling shared unpublished data, M. Godfrey helped interpret TSD output, M. Girondot was extraordinarily helpful with analytical assistance and use of his model, and L. Rodriguez helped with the Spanish translation. This work was permitted under New York State License to Collect and Possess #383 and the Hofstra University Institutional Animal Care and Use Committee.

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Accepted: 12 January 2014.

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