PIVOTAL TEMPERATURE FOR GREEN SEA TURTLES, *CHELONIA MYDAS,* NESTING IN SURINAME

MATTHEW H. GODFREY[1,2] AND N. MROSOVSKY[1]

[1]*Department of Zoology, University of Toronto, Toronto, Ontario M5S 3G5 Canada*

[2]*Present address: Wildlife Resources Commission, North Carolina 28516 USA*

Sexual differentiation of green sea turtles is directed by incubation temperature. The constant incubation temperature that produces both sexes is known as the pivotal temperature, with warmer temperature producing more or all females and cooler temperatures producing more or all males. Here we present data on a laboratory experiment designed to evaluate the pivotal temperature of green turtles from Suriname. The best estimates for the pivotal temperature were 29.4 or 29.5 °C. These values are similar to a previous estimate of pivotal temperature from this green turtle population. When both datasets are combined, the pivotal temperature is estimated to be 29.2 or 29.3 °C. These values are within the range of limited information available from other green turtle populations. Nevertheless, more data from pivotal temperature experiments are needed for a greater understanding of how incubation temperature impacts local nesting populations.

Key words: egg, chelonia, incubation, pivotal temperature, sex ratio, TSD

INTRODUCTION

In many different reptile species, the direction of sexual differentiation is determined by temperature (Shine, 1999). This phenomenon is known as temperature dependent sex determination (TSD) and is often characterized by a pivotal temperature, which is the constant temperature producing equal numbers of each sex, and a transitional range of temperatures or TRT, which consists of those incubation temperatures that produce both sexes (Mrosovsky & Pieau, 1991). The TRT usually spans only a few degrees Celcius, and for incubation temperatures above and below this, all offspring produced are of one sex. All sea turtle species exhibit TSD (Wibbels, 2003). Unfortunately, it is not easy to obtain specific information on pivotal temperatures of sea turtle populations. The expense of precise incubators, the complications of collecting and transporting sea turtle eggs from nesting beaches to laboratory incubators, the labour intensive histology required to classify sex definitively, and the logistical challenge of obtaining permits are some of the disincentives to undertaking a sea turtle pivotal temperature research project. Indeed, despite the discovery of TSD in sea turtles 25 years ago (Yntema & Mrosovsky, 1979), relatively few direct data have been published on pivotal temperatures or TRT for these species (Wibbels, 2003).

Nevertheless, there is a need for more information on pivotal temperatures in sea turtles for a variety of reasons. For instance, there is no validated non-lethal method for classifying the sex of sea turtle hatchlings. Therefore, the sex ratios of offspring produced at a specific sea turtle nesting beach are often derived indirectly by comparing nest temperatures in the field with pivotal temperatures from the laboratory (Baptistotte *et al.*,

1999; Binckley *et al.*, 1998; Broderick *et al.*, 2000; Estes *et al.*, in press; Godley *et al.*, 2001; Godley *et al.*, 2002; Hanson *et al.*, 1998; Mrosovsky *et al.*, 1992; Öz *et al.*, 2004). It is unclear how accurate these estimates are, particularly when nest temperatures are close to pivotal temperatures. Perhaps when nest temperatures are outside of the TRT (e.g. Hanson *et al.*, 1998), the accuracy of estimated sex ratios increases, although few direct validations of the sex ratio estimates of sea turtle hatchling production have been presented to date.

A further problem with comparing nest temperatures with laboratory pivotal temperatures is that TSD data do not exist for all nesting populations of sea turtles. In these cases, researchers often use pivotal temperature data derived from a population elsewhere in the world (e.g. Standora & Spotila, 1985; Baptistotte *et al.*, 1999; Estes *et al.*, in press). Even if data on pivotal temperature are available for the relevant population, they may be based on only one or two clutches of eggs. This may not be a serious drawback if pivotal temperature in sea turtles is a conservative character, as has been suggested (Mrosovsky, 1994). However, so far few data have been available to adequately assess this point.

For these reasons, we present data on pivotal temperature from green sea turtles (*Chelonia mydas*) nesting in Suriname. This nesting population had previously been the focus of several studies concerning offspring sex ratios (Mrosovsky, 1982; Mrosovsky *et al.*, 1984; Godfrey *et al.*, 1996). The present study will increase the database and our knowledge of TSD in sea turtles.

MATERIALS AND METHODS

EGG COLLECTION AND TRANSPORT

The green turtle eggs came from Matapica beach in Suriname (5 59.10N, 54 56.14W). On the night of 8-9 May 1995 eggs were obtained from two separate clutches soon after being laid. At 22.30 hrs local time,

Correspondence: M. H. Godfrey, Wildlife Resources Commission, 1507 Ann St., Beaufort, North Carolina 28516 USA. *E-mail*: mgodfrey@seaturtle.org

90 eggs were taken from one clutch (designated Clutch N), and under supervision of the Surinam conservation officer, the remaining eggs were placed back in the original nest hole. At 00.30 hrs local time, all 108 eggs were taken from another clutch (designated Clutch O). Eggs from each clutch were carefully placed in a Styrofoam box.

The egg boxes were transported on foot for approximately 6 km, then by a small boat to Paramaribo, and finally by plane to Toronto, Canada. Whenever possible, the eggs were kept in air-conditioned rooms or enclosures to provide low temperature during transport (Harry & Limpus, 1989). The eggs arrived in Toronto at 00.30 hrs local time on the night of 9-10 May, and they were unpacked and placed in the incubators by 03.00 hrs local time (i.e. <30 hours after collection from the beach).

LABORATORY INCUBATION

Each egg was randomly assigned a number and placed singly in a 500 ml covered plastic container that contained 60 ml of deionized water, an indented piece of sponge and moistened vermiculite (further details in Mrosovsky, 1988). Up to 16 eggs (in two layers) were placed on either two or three shelves of five incubators set at different temperatures (shelf temperatures given in Table 1). Sixty five ml of deionized water were added to each egg container on days 17 and 41 of incubation.

A glass mercury thermometer (with 0.1 °C scale) encased in a tube of glycerol was placed on each shelf with the thermosensitive bulb roughly in the middle of the shelf. The thermometers were read once daily with an effort to minimize temperature changes in the incubators. To assess evaporative cooling, on day 30 we inserted a needle-thermistor probe into one egg to compare its core temperature to that of an adjacent vial of glycerol. After 24 hrs allowed for equilibration, four readings were taken over two days. The egg core was cooler on average than the glycerol by 0.25 °C. Therefore, a correction factor of 0.25°C was subtracted from all incubator temperature readings to account for evaporative cooling.

After day 45 of incubation, the eggs were checked twice daily for signs of hatching. An egg was considered hatched if the head and at least one flipper of the hatchling were outside of the shell (Godfrey & Mrosovsky, 1997). Incubation duration in the laboratory was calculated as the number of days between laying and hatching. When a hatched egg was found, it was removed from the incubator, the hatchling was quickly killed and the gonads excised and placed in buffered 0.9 % formalin. Following fixation in the formalin for at least seven days, the gonads were prepared as outlined in (Mrosovsky et al., 1984). Briefly, one gonad from each hatchling was cut in half transversely, and embedded in paraffin wax. Serial sections (10 μm thick) were taken from the cut end of the gonad and mounted on slides.

SEXING

The sections were stained with Harris' haemotoxylin and periodic-acid-Schiff reagent (PAS), and examined under a light microscope. Male gonads were characterized by a thin smooth cortex and presence of immature seminiferous tubules; female gonads were characterized by a PAS-positive tunica albuginea between the cortex, which was thickened and infolded, and the medulla, which lacked tubules (for details, see Yntema & Mrosovsky, 1980; Miller & Limpus, 1981). Rarely, some gonads exhibited characteristics of both testes and ovaries. They were labeled intersexes and treated as non-females in the calculations of sex ratio (Pieau et al., 1994). All gonads of embryos that died at a late stage were examined for sex, although usually gonadal tissue from these embryos had degenerated beyond recognition.

Two experienced researchers independently evaluated sections from each gonad. In the rare cases when identification of sex by the two disagreed, the two researchers re-examined the tissue sections together. If consensus could not be reached (this occurred only in a few cases when the samples in question had come from embryos that had died prior to pipping and the tissue had deteriorated), no sex was assigned and those samples were excluded from further analysis.

DATA ANALYSIS

The pivotal temperature was calculated according to three different methods. The first was the simple method, based on taking the data point that falls on the 50% female sex ratio level, or, if such a point is not available, by fitting a straight line to join the two data points that fall closest to the 50% female sex ratio level, and taking pivotal temperature as the point where the line intersected the 50% sex ratio level (Mrosovsky & Pieau, 1991). The second was to use maximum likelihood analysis (TSD software ver. 3.2.2, available at http://www.ese.u-psud.fr/epc/conservation/TSD/index.html) to place a best-fit sigmoidal curve to the data (Girondot, 1999). The third was to fit a sigmoidal

TABLE 1. Constant incubation temperatures (taking into account -0.25 °C correction factor for evaporative cooling) for green turtle eggs from Suriname. [a]includes two unhatched embryos that were able to be classified by sex; [b]includes one intersex (treated as not female)

Temperature ± range °C	Eggs set (N,O clutch)	Eggs hatched (N,O clutch)	Sex ratio (% female)
27.6±0.3	24,24	1,17	0.0[a]
28.2±0.3	14,16	1,9	8.3[a]
28.4±0.3	12,16	1,10	18.2
28.7±0.4	16,16	2,11	35.7[b]
29.4±0.4	16,16	2,11	46.7[a]
30.0±0.5	4,10	0,4	50.0
30.6±0.4	4,10	0,5	100.0

curve to the data using Prism4 (GraphPad, San Diego, Calif.). To determine the pivotal incubation duration (the incubation period that produced equal numbers of both sexes), we also fitted a sigmoidal curve to data on incubation period of eggs vs. sex ratio, using Prism4. For the curve fitting using Prism4, the pivotal results were nearly identical (<0.1 difference) when we used the raw % female data or arcsin transformed data (Zar, 1999).

RESULTS

Only seven out of 90 eggs from Clutch N hatched (7.8% success rate), while Clutch O produced 67 hatchlings (62.0% success rate; Table 1). The overall hatch rates were significantly different between clutches N and O ($\chi^2=59.45$, df=1, $P<0.0001$). Most of the unhatched eggs from Clutch N contained embryos that had died early during development, implying the eggs in the clutch were fertilized. Of the unhatched eggs from clutch N, two were successfully classified by sex; from clutch O, four unhatched eggs were accurately classified
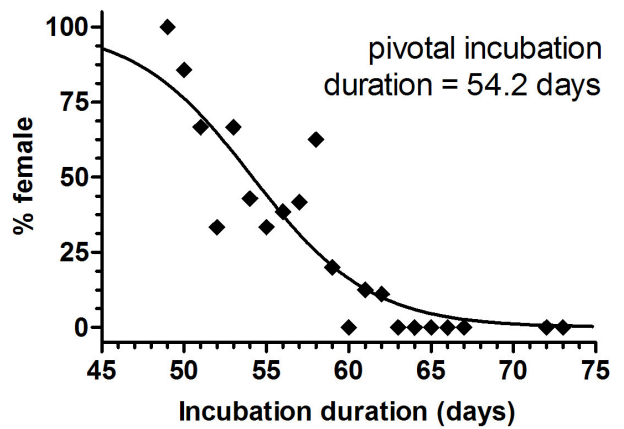
FIG. 2. Relationship between days to hatching and sex ratio in laboratory-incubated green turtle eggs. Data were smoothed using a 2-day running window mean. The sigmoidal curve was fitted to data using Prism4 software.

by sex. One hatchling from clutch O was rated as an intersex. There was no significant correlation between incubation temperature and hatching success of either clutch or both combined (Spearman rank correlation, $P>0.05$).

Some of the different shelves in the incubators had similar incubation temperatures during the experiment. In these cases, the results were pooled (Table 1). Based on the simple method of Mrosovsky & Pieau (1991), the pivotal temperature was 30.0 °C (Fig. 1A). Using Girondot's (1999) method of fitting a sigmoidal curve using maximum likelihood analysis, we derived a pivotal temperature of 29.4±0.1SE °C. When a sigmoidal curve was fit to the data using Prism4 software, the pivotal temperature was estimated to be 29.5±0.1SE °C (Fig. 1A).

Data on incubation period (time to hatching) and corresponding sex ratio of eggs that hatched were smoothed using a 2-day running window mean, and had a sigmoidal curve fitted to them using Prism4 software. The laboratory pivotal incubation duration was 54.2±0.7SE days (Fig. 2).

DISCUSSION

Methods for calculating pivotal temperatures of sea turtles have varied across studies (e.g. Limpus et al., 1985; Mrosovsky, 1988; Chevalier et al., 1999). Indeed, a derived pivotal temperature value from a single dataset can vary 1 °C or more, depending on what procedure is employed (Mrosovsky & Pieau, 1991). In the present case, the small discrepancies in pivotal temperature values among the analyses used were nearly all smaller than the range of constant temperatures recorded during incubation (Table 1). In the 1995 dataset, four eggs incubated at 30 °C produced exactly 50% females. For a point on the steep portion of the sex ratio curve, a sample size of four eggs is too small to be reliable for some methods. If data for that point were excluded, the pivotal temperature would be 29.5 °C, based on the simple method (Fig 1A). This highlights a potential weakness
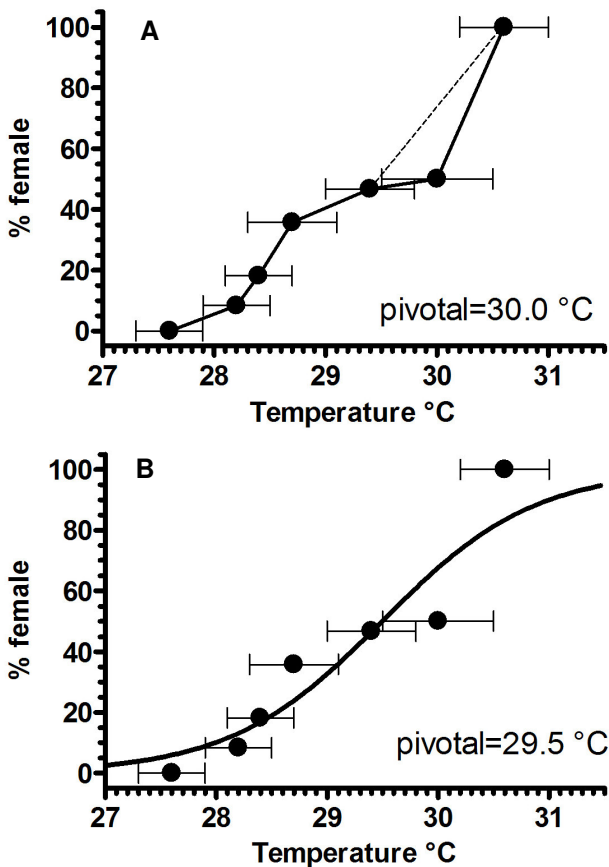

FIG. 1. Relationship between incubation temperature and sex ratio of green turtle eggs from Suriname. (A) shows how the data were plotted for the simple method of calculating pivotal temperature based on Mrosovsky & Pieau (1991). When the sex ratio data from the 30 °C group (n=4) were excluded, the simple method calculated a pivotal temperature of 29.5 °C (dotted line). (B) shows the sigmoidal curve fitted to the data by Prism4 software.

in the simple method of calculating pivotal temperature. Furthermore, the two other methods used to calculate pivotal temperature produced nearly identical values (29.4 and 29.5 °C). Recently developed standardized methods to analyze TSD data from reptiles (Girondot, 1999; Godfrey *et al.*, 2003) facilitate comparisons across TSD studies, although at the current time, we recommend that several methods be used to analyze the same dataset.

The pivotal temperature derived from this study (29.4 or 29.5 °C, depending on the method used) was slightly higher than the 28.8 °C value derived for the same population in an earlier study by Mrosovsky *et al.* (1984). There were some differences in the methods of the two studies; for instance, the earlier study employed a variety of different incubation substrates and also water was added more frequently to the eggs (McLean *et al.*, 1983). Overall, we were more confident of our methods employed in the current study, given its use of a standardized substrate and the wider range of constant incubation temperatures. Nevertheless, both studies were based on a small sample size (150 eggs from three clutches in 1983, 198 eggs from two clutches in 1995), and eggs of one clutch from the present study had a poor survival rate. Therefore, a more representative value of

the pivotal temperature of this green turtle nesting population might be based on a combined analysis of data from both studies. When we reanalyzed the data from both datasets (1983 and 1995), we calculated a pivotal temperature of 29.2 °C based on the simple interpolation method, 29.2±0.1SE °C based on the method of Girondot (1999), and 29.3±0.2SE °C, based on a sigmoidal curve fit with Prism4 (Fig. 3). Given the small discrepancy among the three methods to calculate pivotal temperature, 29.2 °C is the best estimate for this population at the current time.

Pivotal temperatures have been derived for some nesting populations of green turtles from around the world, using different methods of egg incubation (Table 2). Studying the sex ratio of hatchlings relative to nest temperatures on nesting beaches is valuable for gaining insight into the micro-environemental conditions specific to the study beach. However, studies involving field incubation of eggs for pivotal temperatures vary in methodology, particularly in terms of impact of substrate, hydric conditions, and nonrandom collection of a subset of hatchlings for classification of sex. This makes it difficult to compare pivotal temperatures across studies and populations. In contrast, laboratory incubation of eggs at constant temperatures is valuable as a more standard method that facilitates comparisons of results. Data from constant temperature experiments are also useful in developing "constant temperature equivalents" for interpreting variable temperature regimes recorded in natural conditions relative to hatchling sex (Georges *et al.*, 2004). Additionally, pivotal temperatures based on laboratory incubation and extrapolated sex ratios are basic life history characters that can be used in larger scale analyses, such as modeling of population dynamics (Crouse *et al.*, 1987; Chaloupka, 2002) and predicting population responses to environmental pressures such as global climate change (Mrosovsky *et al.*, 1984; Davenport, 1989).

Nevertheless, laboratory incubation of eggs remains far from being completely standardized among researchers and laboratories. For example, some researchers take into account evaporative cooling of eggs when presenting laboratory incubation temperature data (e.g. Mrosovsky, 1988; Godfrey *et al.*, 1999; this study) while others do not (e.g. Binckley *et al.*, 1998; Georges *et al.*, 1994; Wibbels *et al.*, 1998). Furthermore, some incubators used in the laboratory may have wide ranges around the mean "constant" temperature. For instance, a standard deviation of up to ±0.5 °C around the mean incubator temperature may translate into a temperature range of several °C (Limpus *et al.*, 1985). Cyclical temperature fluctuations about a mean temperature can, if large enough, result in more female hatchlings being produced than predicted by the mean non-fluctuating temperature (Georges *et al.*, 1994). A wide range of temperatures around the mean may explain the low pivotal temperature of <29 °C reported by (Miller & Limpus, 1981) in Table 2. Finally, there may also be differences across studies in the humidity levels
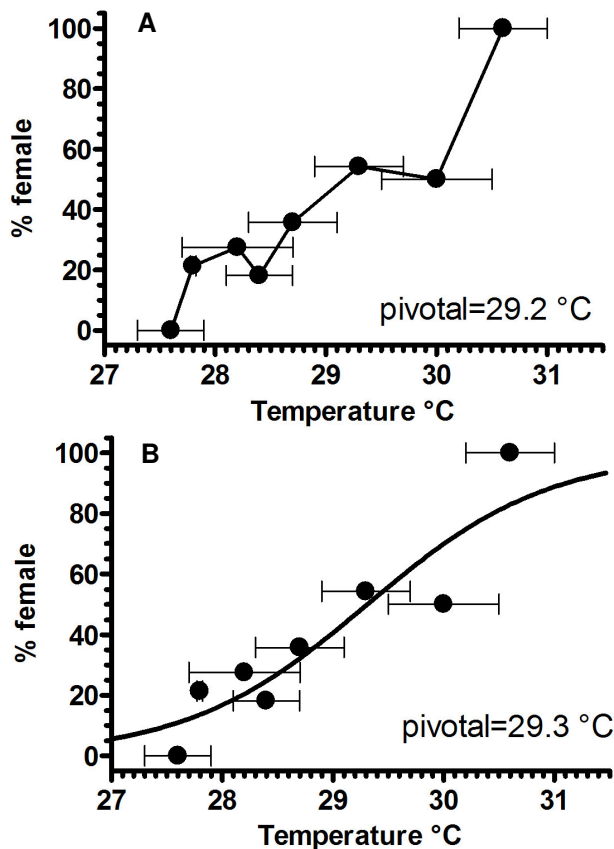

FIG. 3. Relationship between incubation temperature and sex ratio of green turtle eggs from Suriname, combining data from 1983 (Mrosovsky *et al.*, 1984) and 1995 (this study). (A) shows how the data were plotted for the simple method of calculating pivotal temperature based on Mrosovsky & Pieau (1991). (B) shows the sigmoidal curve fitted to the data by Prism4 software.

of the incubators or incubating substrate that can also affect sex during incubation (Steyermark, 1999), although the impact of thermal differences generally far outweighs those of nonthermal differences in incubation (Godfrey & Mrosovsky, 2001). Perhaps the best comparisons of pivotal temperatures across studies are restricted to data that are collected by the same laboratory using the same methodology. To date, in the laboratory used for the present study on green turtles, five other incubation studies have been conducted using nearly identical methods on clutches from: loggerhead turtles from USA, Brazil, and Greece (Mrosovsky, 1988; Marcovaldi et al., 1997; Mrosovsky et al., 2003), and hawksbill turtles from Antigua and Brazil (Mrosovsky et al., 1992; Godfrey et al., 1999). Interestingly, the pivotal temperatures from all these studies and species have been close to 29 °C, which conforms to the idea that pivotal temperatures in sea turtles are constrained around 29 °C (Mrosovsky, 1994).

However, there remain some qualifications of this claim of conservatism around pivotal temperatures in sea turtles. First, there are relatively few pivotal temperature data available to date, and the majority come from loggerhead sea turtles. It may be the case that in loggerheads, there is conservatism of pivotal temperatures around 29 °C, but the scant data available for species such as hawksbills, flatbacks, and green turtles make generalizations tenuous. Second, recent data from olive ridley sea turtles from Pacific Costa Rica reported that the pivotal temperature for this population is close to 31 °C (Wibbels et al., 1998). Interestingly, a study on olive ridleys from India found a pivotal temperature of 29.5 °C (Mohanty-Hejmadi & Dimond, 1986). This could signal a wider variation of pivotal temperatures in olive ridleys or even other sea turtle species. However, it must be kept in mind that methodological variation in

the incubation methods may account for some or all of this higher pivotal temperature. For instance, there was no correction for evaporative cooling by (Wibbels et al., 1998), although even adding a correction factor of -0.5 °C would still result in the highest pivotal temperature for sea turtles (Wibbels et al., 1998). Also, the olive ridley eggs were incubated in groups rather than singly, which may have affected the metabolic warming produced during incubation. A better comparative test would be to incubate eggs of two or more different species and/or populations simultaneously in the same incubators using the same methodology.

Overall, there remains much work to be done on TSD in sea turtles, particularly in uncovering the nature of pivotal temperatures of different populations and species. We urge that more effort be spent on building up the database on pivotal temperatures. These data have a wider application, including forming a natural history character that can be used in population modeling and contributing to research about evolution (Rhen & Lang, 1998; Girondot & Pieau, 1999). They also have application for management and conservation, in terms of estimating the potential impacts on sea turtle sex ratios not only by local changes in nesting habitats (e.g. Mrosovsky et al., 1995), but also by long term impacts such as increased feminization due to global climate change or conservation efforts (Janzen, 1994; Girondot et al., 1998). Also important is the need to have data on TSD relevant to the local sea turtle population, rather than imported data from other regions or even ocean basins.

ACKNOWLEDGEMENTS

TABLE 2. Values of green turtle pivotal temperatures from various populations, based on individual studies

Population	Pivotal temperature ^{0}C	Clutches	Method to estimate pivotal	Reference
LABORATORY STUDIES				
Suriname (1995)	30.0 (or 29.5)	2	simple method	This study
Suriname (1995)	29.5±0.2SE	2	sigmoidal curve fit (Prism4)	This study
Suriname (1995)	29.4±0.1SE	2	maximum likelihood curve fit	This study
Suriname (1983)	28.8	3	straight line	Mrosovsky et al., 1984
Suriname (1983&1985)	29.2	5	simple method	This study
Suriname (1983&1985)	29.3±0.2SE	5	sigmoidal curve fit (Prism4)	This study
Suriname (1983&1985)	29.2±0.1SE	5	maximum likelihood curve fit	This study
Sarawak	<29.5	11	extrapolation	Leh et al., 1985
Australia	<29.0	1	straight line	Miller & Limpus, 1981
FIELD STUDIES				
Costa Rica	28.0 to 30.3	33	unknown	Standora & Spotila, 1985
Ascension Is.	28.8	21	maximum likelihood curve fit	Godley et al., 2002
N. Cyprus & Turkey	28.7	5	regression line	Kaska et al., 1998
N. Cyprus	28.7, 29.2	48	regression line	Broderick et al., 2000

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