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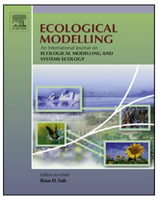

Sex ratio estimates for species with temperature-dependent sex determination differ according to the proxy used

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ABSTRACT

Knowledge of the sex ratio of a population is crucial to understand their structure and dynamics. For species, such as marine turtles, with temperature-dependent sex determination, this knowledge provides a baseline in advance of climate change. Determining the primary sex ratio for marine turtle populations is challenging since offspring lack sexually dimorphic external characteristics. Therefore several proxies have been used to estimate the primary sex ratio of marine turtle populations. However, no study to date has compared estimations of sex ratio when using different proxies to determine the most accurate and to detect potential bias. To address this, we estimated the sex ratio of natural loggerhead, *Caretta caretta,* nests using 8 different proxies: two based on constant temperature equivalent (average of temperature or average temperature weighted by the growth of embryos during each time step) both for three developmental periods (the whole incubation, the middle third of incubation and the middle third of development) as well as two proxies based on incubation duration (duration of the whole incubation and of the middle third of development). Sex ratio estimates differed greatly depending on the proxy being used. Here we discuss the differences among proxies based on the biological relevance of underlying hypotheses and highlight the need for studies to accurately determine the thermosensitive period and to obtain appropriate estimates of embryo growth rate to estimate marine turtle sex ratio.

1. Introduction

Knowledge of the sex ratio of a population is crucial to understand their structure, dynamics and their evolutionary potential; this informs population viability analysis and effective population size calculations, are relevant to the management and conservation of species (Brook et al., 2000; Ewen et al., 2001). An understanding of primary sex ratio is also important in the context of a warming climate, where populations of species with temperature-dependent sex determination (TSD), may have their sex ratios altered with changes in temperatures (Stubbs et al., 2014; dei Marcovaldi et al., 2016).

Determining the primary sex ratio of populations can be challenging, especially for species that lack heteromorphic sex chromosomes and when offspring lack sexually dimorphic external characteristics. This is the case for marine turtles which exhibit TSD, where incubation temperature triggers the development of either testes or ovaries in embryos (Yntema and Mrosovsky 1980; Mrosovsky 1982; Janzen and Paukstis 1991), and they are not externally dimorphic until puberty (Miller 1997; Wyneken 2001; Ceriani and Wyneken 2008). Thus, reliable sexing of marine turtle hatchlings require the sacrifice of animals and histological examination of the gonad, which is difficult to justify for threatened species

Since empirical estimates of *in situ* primary sex ratios are difficult to obtain, several proxies (e.g., incubation temperature and duration) have been used to estimate the primary sex ratio of marine turtle populations (Girondot et al., 2010; Wyneken and Lolavar 2015). Wyneken and Lolavar (2015) recently categorized then based on how many steps are necessary to determine sex from the actual embryonic sex ratio, with an increase in proxy "distance" decreasing the reliability of the sex ratio estimate. First level proxies includes samples of hatchlings from nests, second level proxies includes the use of incubation temperature during the Thermosensitive Period (TSP) – developmental period for which temperature influences gonadal differentiation (Mrosovsky and Pieau 1991), and

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Table 1
Description of each proxy. Proxies 1–3 are averaged temperature CTE_A, proxies 4–6 are averaged temperature weighted by the growth of embryos CTE_W and proxies 7 and 8 are based on the duration of two developmental periods, respectively the whole incubation period and the middle third of development which corresponds to the thermosensitive period for sex determination (named TSP in this study).

Proxy ID	Proxy base	Temporal scale	Biological assumptions
1	Constant Temperature Equivalent averaged temperature (CTE_A) in °C	Whole incubation	Sex is determined only by temperature and during the whole incubation period.
2	Constant Temperature Equivalent averaged temperature (CTE_A) in °C	Middle third of incubation	Sex is determined only by temperature and only during the middle third of incubation period.
3	Constant Temperature Equivalent averaged temperature (CTE_A) in °C	Middle-third of development (TSP)	Sex is determined only by temperature and only during the thermosensitive period for sex determination (TSP).
4	Constant Temperature Equivalent averaged temperature weighted by the growth of embryos (CTE_W) in °C	Whole incubation	Sex is determined by the interaction between temperature and growth rate and during the whole incubation period.
5	Constant Temperature Equivalent averaged temperature weighted by the growth of embryos (CTE_W) in °C	Middle third of incubation	Sex is determined by the interaction between temperature and growth rate and only during the middle third of incubation period.
6	Constant Temperature Equivalent averaged temperature weighted by the growth of embryos (CTE_W) in °C	Middle-third of development (TSP)	Sex is determined by the interaction between temperature and growth rate and only during the thermosensitive period for sex determination (TSP).
7	Duration-based equivalent in days	Whole incubation (linearly proportional with the middle third of incubation)	Indirect measure of the effect of the thermal environment on both growth and gonadal differentiation throughout the whole incubation period.
8	Duration-based equivalent in days	Middle-third of development (TSP)	Indirect measure of the effect of the thermal environment on both growth and gonadal differentiation throughout the thermosensitive period for sex determination (TSP).

third level proxies includes, among others, incubation duration to infer TSP, the use of Constant Temperature Equivalent (CTE) to transform field incubation temperatures to a constant temperature that would result in the same developmental rate to determine sex ratio based on laboratory curves, and the use of climatic data to infer nest temperatures. It has been widely highlighted that these proxies need to be validated with actual sex ratio expressed in nests and that caution is necessary when interpreting sex ratio estimates from proxies (Girondot et al., 2010; Wyneken and Lolavar 2015). Unfortunately, for ethical, logistical and economic reasons validation is challenging (Gross et al., 1995; Wyneken et al., 2007), and consequently several studies have used and continue to use these proxies. Even though these proxies have been reviewed separately, no study to date has compared estimations of sex ratio when using different proxies to determine the most pertinent and detect potential bias. To address this, we estimated the sex ratio of natural loggerhead, *Caretta caretta,* nests using 8 different proxies and discuss the differences among methods based on the biological relevance of underlying hypotheses.

2. Materials and methods

2.1. Incubation temperatures and durations from field

The temperature of 43 nests of loggerhead turtles at Praia de Forte, Bahia, Brazil was recorded during the 2014–2015 nesting season (21st October 2014 to the 11th of March 2015). Five of them were removed from analysis because they showed abnormal temperature patterns that were likely due to predation and consequent exposure events. This region is an important loggerhead nesting area for the Southwest Atlantic loggerhead Regional Management Unit (Wallace et al., 2010) and an intensive study area for TAMAR (for information on TAMAR, see Marcovaldi and de Marcovaldi 1999). Of the 38 nests used for this study, 16 nests were monitored using Minilog data loggers (VEMCO Minilog-II-T, accuracy ± 0.1 °C, resolution = 0.01 °C) and 22 others with Thermochron iButtons (DS1922L-F5, accuracy ± 0.5 °C, resolution = 0.0625 °C). Dataloggers were placed at the middle of the clutches while females laid their eggs and removed just after hatchlings emerged. Incubation durations, are defined here as the time between when the eggs were laid and when the first hatchlings emerged. Air temperature at 2 m above the ground level and sea surface temperature in front of the beach were retrieved from the European Centre for Medium-range Weather Forecasts (ECMWF) which provide a global reanalysis of climate temperature for the last 37 years on earth at a 0.125° spatial resolution (ERA-Interim project), where models used historical records to provide smoothed temperature time hindcasts. We retrieved data every 6 h from the 1st January 1979 to the 31th December 2015 (latitude 12.375°S and longitude 38°W).

Temperature time series recorded within natural nests can be viewed as an autocorrelative process by which temperature recorded at time i depends on temperature at time i-1. Statistical properties of such time series are analyzed by autoregressive moving-average (ARMA) models which describe stationary stochastic process using two parameters, p and q (p is the lag for the coefficient of auto regression AR and q is the lag for the moving average MA). The ARMA model that best describe daily nest temperature was estimated for p = 0 or 1 and q = 0 or 1 (with 0 being time i and 1 being time i-1). We summed the Akaike Information Criterion (AIC) for each of the 38 temperature time series recorded in natural conditions for each of these p and q combinations. AICs are then compared to test which ARMA model best describes statistical properties of nest temperature time series.

2.2. Proxies for sex ratio estimations

The primary sex ratio, for each monitored nest, was predicted using 8 different proxies: 2 based on constant temperature equivalent average of temperature or average temperature weighted by the growth of embryos (in term of embryo size measured by straight carapace length during each time step) both for 3 developmental periods (the whole incubation, the middle third of incubation and the middle third of development) as well as 2 proxies based on incubation duration (duration of the whole incubation and of the middle third of development) (Table 1).

2.2.1. Temperature-based proxies (proxies 1–6)

The relationship between marine turtle sex ratio and temperature or incubation duration (reaction norm of sex ratio) is first obtained with incubation experiments at constant temperature in laboratory conditions. This reaction norm is then applied to measures obtained in the field for the same population (see (Marcovaldi et al., 1997; The Chu et al., 2008; dei Marcovaldi et al., 2014; dei Marcovaldi et al., 2016)). Indeed for the population studied here, this relationship was determined with two clutches collected in 1995 and incubated artificially at 6 constant temperatures ranging from 27.4 to 30.6 °C (Marcovaldi et al., 1997). However, in natural conditions, incubation temperature fluctuates according to circadian cycle or from day to day, and is not constant. Therefore, temperature time series recorded within nests cannot be used as such to predict sex ratio from the relationship established at constant temperatures. Thus, to increase the accuracy of sex ratio estimates from nest profiles, a Constant Temperature Equivalent (CTE) has been used for different incubation periods to determine sex ratios (see (Georges et al., 1994; Georges et al., 2005; The Chu et al., 2008; Woolgar et al., 2013)). It has been proposed that average incubation temperature during any developmental periods (named here CTE_A) is not able to reflect correctly the effect of temperature on gonadal differentiation (Valenzuela 2001). This is because the growth of embryos (or cell proliferation) is also dependent on temperature. In other terms, the higher the temperature (which is more likely feminizing) the faster the growth and the lower the temperature (which is more likely masculinizing) the slower the growth. Considering this, embryos that spend equal time at masculinizing and feminizing temperatures would develop into females because more cells have proliferated during the feminizing period rather than during the masculinizing one. CTE weighted by embryonic growth (named here CTE_W) is another type of proxy that accounts for the rate of gonadal differentiation according to the differential growth of embryos at different temperatures. Here we propose to calculate CTE_W by multiplying the incubation temperature by the fraction of development realized for each period from k to N:

$$CTE = \frac{\sum_{i=k}^{N-1} T_i \times W_i}{\sum_{i=k}^{N-1} W_i} \tag{1}$$

Where T_i is the temperature at the moment i during incubation with k being the first recorded temperature during the considered developmental period. W_i is the growth of embryos in term of straight carapace length (SCL) between two records (i.e. the difference between the embryonic size at time i + 1 and i). N is the last recorded temperature during the considered developmental period. The following developmental periods were considered for temperature-based proxies (CTE_A and CTE_W – proxies 1–6, Table 1): (i) the total incubation duration, (ii) the middle third of incubation period and (iii) the middle third of development (which corresponds to the thermosensitive period for sex determination and named TSP in this study- as determined in section 2.3) for each of the 38 natural nests monitored.

2.2.2. Duration based proxies (proxies 7 and 8)

Temperature has a direct effect on marine turtle embryonic growth and thus on the timing to complete embryonic development (Miller 1985). Consequently, the duration of the total incubation period (from a freshly laid egg to a completely developed embryo) is an integrated measure of thermal incubation conditions. Given this, incubation durations recorded in the field (from nest laying date to hatching date) have been used as a proxy to predict sex ratio of clutches (Fuller et al., 2013; dei Marcovaldi et al., 2014; Jribi and Bradai 2014; dei Marcovaldi et al., 2016). However, given daily variability in thermal fluctuations in natural conditions, the total incubation duration does not necessarily reflect thermal conditions during the TSP. The TSP occurs during incubation when gonads form and until a critical threshold when gonads are sufficiently developed to differentiate along the male or female pathways. This period corresponds to the middle third of development (Mrosovsky and Pieau 1991). Thus, the progression of embryonic development during incubation needs to be modeled to access information about TSP boundaries during incubation and then its duration.

2.3. Modeling embryonic development

To determine sex ratio for proxies 3, 6, and 8, which are related to the middle third of development, the growth of embryos throughout incubation needed to be calculated to calculate the TSP boundaries. The change of embryonic size can be modeled using a growth function where the growth rate depends on temperature (see section 2.3.1). Therefore, the thermal reaction norm for growth rate (i.e. the pattern of growth rate across a range of temperature) needs to be estimated to model the growth of embryos accounting for such phenotypic plasticity. Here we modeled both the thermal reaction norm for growth rate and the progression of embryonic size in term of straight carapace length (SCL) for the 38 natural nests. Thereafter, we determined TSP boundaries and incubation duration for each nest. We used SCL metric rather than mass to ensure that the first-order derivative of the chosen metric can never be negative as does the Gompertz equation (see section 2.3.1).

2.3.1. Progression of straight carapace length during incubation

Thermal reaction norm for growth rate can be estimated from traces of temperature recorded within natural nests (Girondot and Kaska 2014). Here we used the 4-parameters equation to describe the effect of temperature on the embryonic growth rate for loggerhead turtles as per (Girondot and Kaska 2014). The equation is given below:

$$r(T) = \frac{\rho_{(298\mathrm{K})}\frac{T}{298}exp\left[\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{298}-\frac{1}{T}\right)\right]}{1+exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}}-\frac{1}{T}\right)\right]} \tag{2}$$

Where $r(T)$ is the rate of embryonic growth at temperature T (time^{-1}), R is the universal gas constant ($\mathrm{J\,K^{-1}\,mol^{-1}}$),$T_{1/2_H}$ is the is the temperature in K at which the enzymes are 1/2 active and 1/2 inactive due to high-temperature effect, ΔH_H is the change in enthalpy associated with the high-temperature inactivation of the enzymes ($\mathrm{J\,mol^{-1}}$), $\Delta H_A^{\neq}$ is the enthalpy of activation of the reaction catalyzed by the enzymes ($\mathrm{J\,mol^{-1}}$) and $\rho_{(298\mathrm{K})}$ is the developmental rate at 24.85 °C (298 K) assuming no enzyme inactivation (time^{-1}). Thus the fitted variables are $\rho_{(298\mathrm{K})}T_{1/2_H}$, ΔH_H, $\Delta H_A^{\neq}$ and

The change of size of embryos upon incubation time is described by the Gompertz equation which have been modified by (Laird 1964):

$$X(t) = K\exp\left(ln\left(\frac{X(0)}{K}\right)exp\left(-r(T)\,t\right)\right) \tag{3}$$

Where $X(0)$ is the size of a gastrula at nesting time (time = 0), $r(T)$ is the growth rate and K is the asymptotic value when $t \to \infty$. $X(t)$ is the size of embryos at any time during incubation and depends on the rate of growth which depends on temperature. To inform this, we measured a subset of hatchlings (n = 70) from one monitored clutch at Praia do Forte during the study period. The straight carapace length of hatchlings was measured, and an average size of 40.97 mm (SD 0.96 mm) was recorded. The confidence interval of the thermal reaction norm for growth rate has been obtained by simulating the posterior distribution of the four parameters using

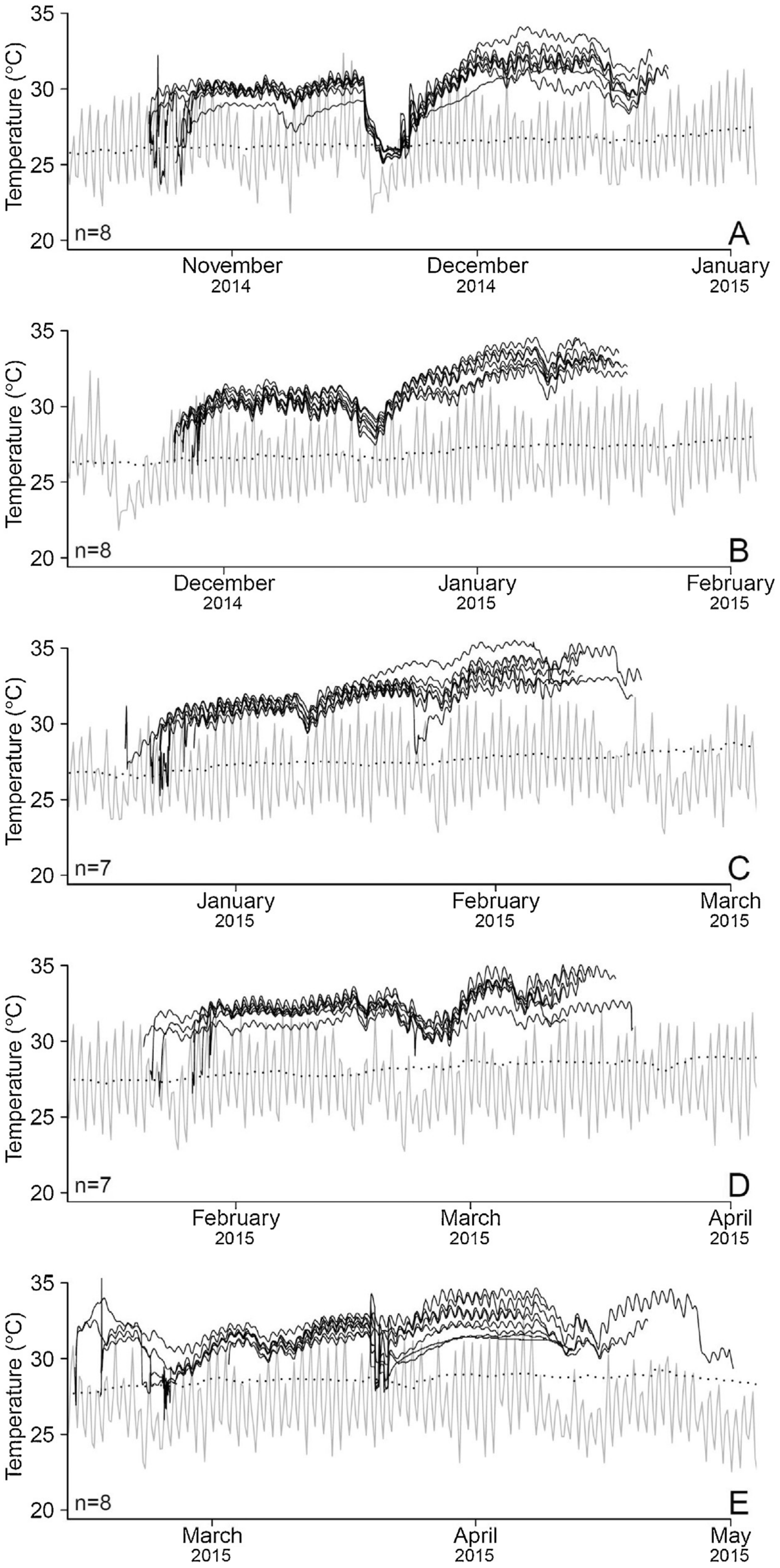

Fig. 1. Variation in incubation temperature for 38 natural nests (black lines), along with: air temperature at 2 m above the surface (grey lines) and sea surface temperature in front of the nesting beach (dotted lines). Nests were laid in A) October 2014, B) November 2014, C) December 2014, D) January 2015, and E) February 2015. Note that time frames for each plot are shifted by one month depending when the nest was laid.

MCMC with Metropolis-Hastings algorithm (Chib and Greenberg 1995) during 100 000 iterations. An adaptive algorithm has been used to satisfy the acceptance rate close to 0.234 by adjusting the distributions of the new proposals every 100 iterations (Rosenthal 2011). Initial parameter values during the MCMC search corresponded to the set of values that maximized likelihood (for more

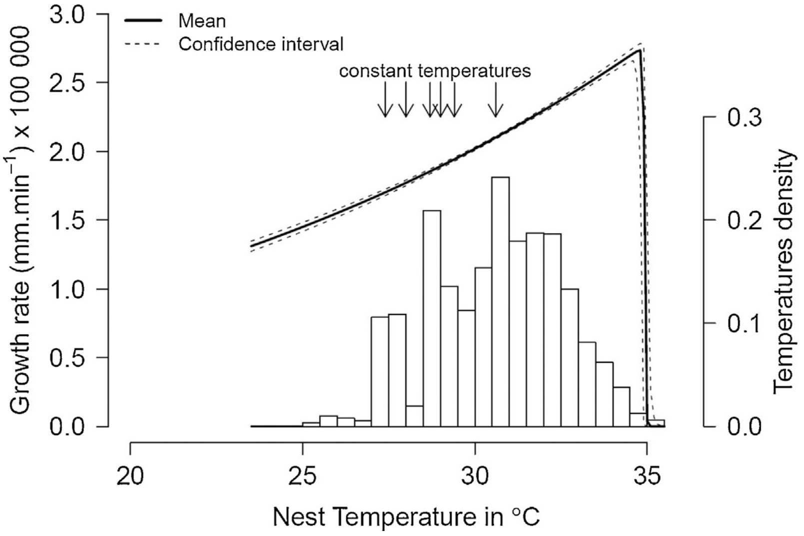

Fig. 2. Thermal reaction norm for embryonic growth rate for Brazilian loggerhead population nesting at Praia de Forte. The confidence interval (dotted lines) is based on quantiles 2.5% and 97.5%.

details see Girondot and Kaska 2014). The priors are $U\,(500)$ for $\Delta H_A^{\neq}$, $U\,(100000)$ for ΔH_H, $U\,(500)$ for $T_{1/2_H}\rho_{(298\mathrm{K})}$ and $N\,(308;\,7)$ for

The thermal reaction norm model was informed by the 38 temperature times series recorded in the field at Praia de Forte and the 12 constant temperature experiments from Marcovaldi et al. (1997).

2.3.2. Determining period of development (whole incubation, the middle-third of incubation duration, and the middle-third of development)

CTEs (proxies 1–6) were implemented for three incubating periods (i) the whole incubation period, (ii) the middle third of incubation period and (iii) the middle-third of development (TSP), and the duration-based proxies (proxies 7 and 8) were implemented for (i) the whole incubation duration and (ii) the middle third of development. We did not calculate the duration of the middle third of incubation as a third duration-based proxy because this measure is directly proportional to the whole incubation duration and would therefore provide redundant results.

The whole incubation period is defined from a freshly laid egg at gastrula stage to a completely developed at hatchling. The middle third of incubation starts at the 1/3 of the whole incubation duration and ends at 2/3 of the whole incubation duration. The TSP starts when embryos reach 1/3 of their final size before hatching and ends when embryos reach 2/3 of their final size.

2.4. Reaction norm of sex ratio at constant temperature

Loggerhead turtles species exhibits a male/female pattern of TSD which corresponds to the production of males at low temperatures and females at high temperatures (see (Wibbels 2003)for a review). For the population in focus here, their sex ratio determined histologically, the constant incubation temperature and the total incubation duration have been previously established for 185 hatchlings from two nests (Marcovaldi et al., 1997). We modeled the embryonic development (as described in section 2.3.1) for these 6 constant incubation temperatures and calculate the duration of the TSP.

The sex ratio thermal reaction norm can be modeled using several equations whereas the global pattern is summarized by the pivotal temperature at which sex ratio is equal to 0.5 (P) and the range of temperature within which both sexes are produced is called the Transitional Range of Temperatures (TRT). The relationship between sex ratio and incubation duration can be modeled using similar equations and it permits to define the pivotal duration (PD) at which sex ratio is equal to 0.5 and the range of duration within which both sexes are produced is called the Transitional Range of Durations (TRD). It should be noted that the pattern describing sex ratio versus temperature is reversed as compared to the pattern of sex ratio versus incubation duration. Indeed, embryos that incubate at high temperature develop faster and are more feminized than those incubated at cooler temperature. However, this is true only for monotonic relationship between temperature and rate of development. This relationship cannot be used if incubation duration slowdown at very high temperature.

Genotypic Sex Determination (GSD) is modeled as a constant 0.5 sex ratio for all temperatures or durations. Four other equations can be fitted if sex is determined by temperature (For more detailed see Hulin et al., 2009). The simplest equation is the logistic model:

$$sr\,(proxy) = \frac{1}{1 + \mathrm{e}^{\left(\frac{(P - proxy)}{S}\right)}} \tag{4}$$

Where, $sr\,(proxy)$, is the sex ratio (here the proportion of males) in terms of the considered proxy. This equation can be modeled to account for a logarithmic scaling in the shape of the transition between the production of 100% males and 100% females. This model is the Hill equation:

$$sr\,(proxy) = \frac{1}{1 + \mathrm{e}^{\left(\frac{(\log P - \log proxy)}{S}\right)}} \tag{5}$$

To account for a potential asymmetrical transition in the sex ratio produced around the pivotal value of the considered proxy, a third parameter K can be introduced. This model is the Richards equation:

$$sr\,(proxy) = \left[\frac{1}{1 + \left(2\mathrm{e}^{K} - 1\right)\mathrm{e}^{\left(\frac{(P - proxy)}{S}\right)}}\right]^{\frac{1}{e^{K}}} \tag{6}$$

In this equation, the parameter K does not depend on the value of the proxy. Given this, a sharp transition before the inflection point of the sigmoid curve implies a smooth transition after and vice versa. To account for an asymmetrical pattern with a potential similar shape above and below the pivotal value of the proxy, two

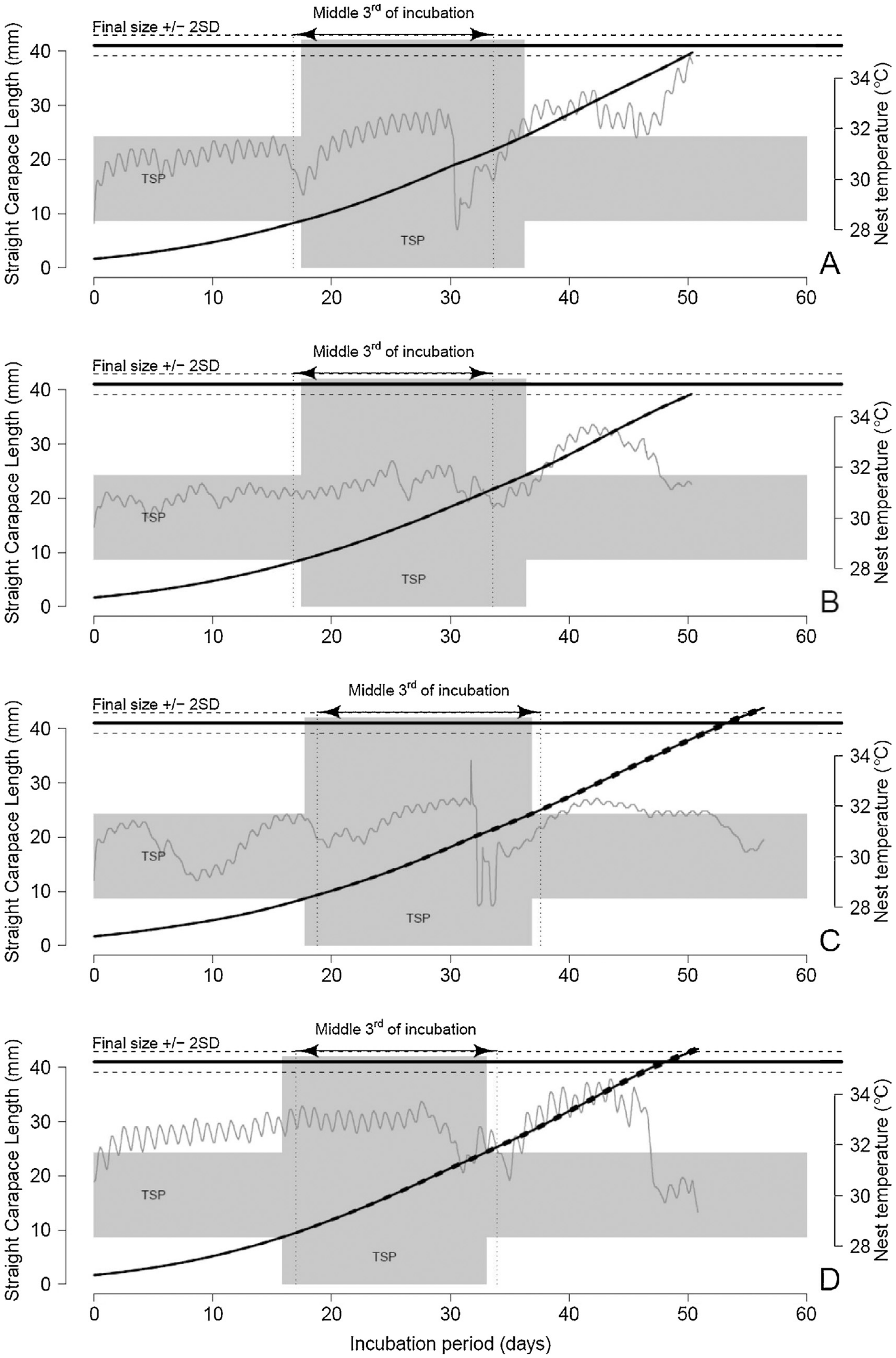

Fig. 3. Model of embryonic development for 4 nests. Nests were laid A) December 2014, B) January 2015, C) February 2015, and D) March 2015. For comparison the nest with the most delayed thermosensitive period (TSP) compared to the middle third of incubation were plotted to illustrate the model of embryonic growth (black line). Dotted lines around this curve represent the confidence interval. Grey lines show traces of incubation temperature. The boundaries of the TSP during development (left y axis) and the matching period during incubation (x axis) are indicated by the grey area. The horizontal black line represents the final straight carapace length (SCL) which is 40.97 mm and the horizontal dotted lines show ±2 sd=0.96 mm. Vertical dotted lines represent the boundaries of the middle third of incubation.

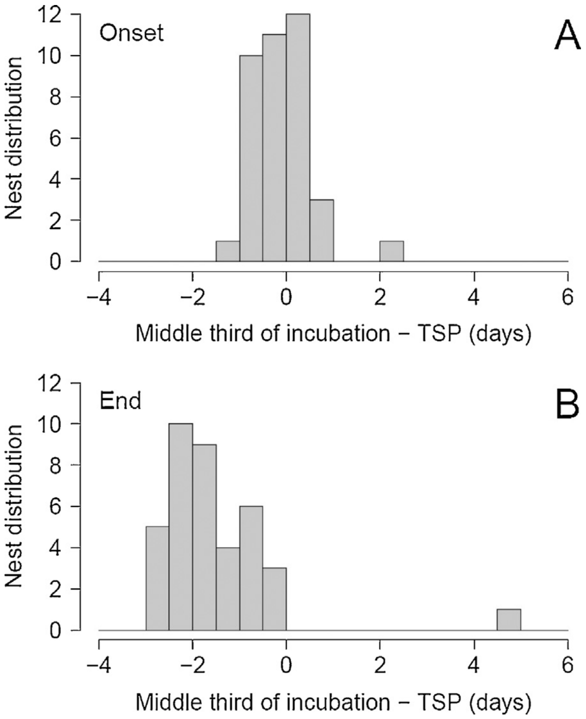

Fig. 4. Shifting of the thermosensitive period for sex determination (TSP) in natural condition. Relative frequency of differences between the onset of the middle third of incubation and the onset of the TSP (A) and between the end of the middle third of incubation and the end of the TSP (B) in days for the 38 natural nests.

parameters K_1 and K_2 replace the previous one. This equation is named Hulin model is:

$$sr\,(proxy) = \left[\frac{1}{1 + \left(2e^{K_1.proxy+K_2} - 1\right) e^{\left(\frac{(P-proxy)}{S}\right)}} \right]^{\frac{1}{e^{K_1.proxy+K_2}}} \tag{7}$$

To select the most appropriate equation/model that describes the relationship between sex ratio and the considered proxy, we compared their Akaike information criteria (AIC) with a correction for finite sample sizes (AICc) using the Akaike weight which gives relative support for each model (Burnham and Anderson 2002).

This model selection was conducted independently for sex ratio against (i) temperature to be used with proxies 1–6, (ii) incubation duration for proxy 7, and (iii) TSP duration for proxy 8. The 3 resulting equations are used further to predict the sex ratio of natural nests using each proxy.

2.5. Quantifying distances among sex ratio estimates using different proxies

Once sex ratio estimates have been obtained using each proxy (proxy 1–8), we calculated the Euclidean distance for each pairwise comparison of proxy-specific sex ratio estimates in order to quantify differences among proxies. Those distances are then clustered using unweighted pair group method (UPGMA). Thereafter, results are represented using a dendrogram to visualize distances among proxy-specific sex ratio estimates.

3. Results

3.1. Thermal incubating environment

Temperature recorded within nests ranged from 23.6 °C to 35.5 °C (mean=31.34 °C, sd = 1.62 °C) with an average daily amplitude of 0.61 °C (Fig. 1). Temperature recorded within nests was higher than air temperature and sea surface temperature.

Table 2
Model selection for the equation that best describes the relationship between the true sex ratio observed in laboratory conditions and the considered proxy. Lowest AICc values are indicated in bold and the Akaike weight in brackets.

Equation/model	Incubation durations	TSP durations	Constant temperatures
Logistic	28.15	28.08	**28.48** (0.50)
Hill	**27.95** (0.52)	**27.91** (0.51)	28.50
Richards	34.80	35.00	36.28
Hulin	62.80	63.03	64.54
GSD	163.41	163.41	163.41

Although a temporal trend of sea surface temperature and nest temperature shows a slight increase along the nesting period (October to March), air temperature did not show a clear seasonal trend within this temporal window (Fig. 1).

Four ARMA models have been fitted to nest temperature time series (with p=0 or 1 and q=0 or 1). The lowest AIC is observed for the ARMA model with p=1 and q=0 (AIC=36355.33) as compared to the following models: p=0 and q=0 (AIC=65615.86), p=0 and q=1 (AIC=56357.71) and p=1 and q=1 (AIC=36396.15). This suggest that nest temperature time series can be viewed as an autoregressive process of first order. In other words, temperature at time t is correlated with temperature at time t-1 and no moving average component is needed.

3.2. Embryonic development model

The thermal reaction norm for embryonic growth rate shows a non-monotonic pattern where the growth rate increases with temperature to reach a maximum at 34.5 °C. The embryonic growth rate at 34.5 °C is 2.7 times higher than at 20 °C and decreases sharply when nest temperature exceeds 34.5 °C (Fig. 2).

TSP durations varied from 20.94 days to 22.57 days for nests which have been laid during October 2014 and from 17.17 days to 20.02 days for those after mid-November 2014 (Appendix A in Supplementary material). The TSP does not always fit with the middle third of incubation in natural conditions (Fig. 3). For instance, the TSP is delayed from up to 2 days (Fig. 3A and B) or it starts earlier (Fig. 3C and D) as compared to the middle third of incubation period for a set of 4 nests which have incubated after mid-December 2014.

We calculated the difference between the timing for the onset of the middle third of incubation and the onset of the TSP (Fig. 4A) and between the timing for the end of the middle third of incubation and the end of the TSP (Fig. 4B) for all natural nests. The TSP can start 2 days before the onset of the middle third of incubation or 1.5 days after (Fig. 4A). For all nests except one, the TSP ends after the end of the middle third of incubation with a delay that reaches from 1 to 3 days for 74% of nests (Fig. 4B).

A difference of 4 days between the duration of the TSP and the duration of the middle third of incubation results in 20% discrepancy if the TSP lasts 20 days and 27% discrepancy if the TSP lasts 15 days.

3.3. Reaction norm of sex ratio at constant temperatures

The logistic model was selected as the best equation of thermal reaction norm of sex ratio for proxies 1–6 to be used (P=29.12 °C, S=-0.27, Transitional Range of Temperature=29.96 to 28.31 °C, Fig. 5A), whereas the Hill model was selected as the best equation for the incubation duration proxies (proxy 7) (P=59.32 days, S=0.019, Transitional Range of whole incubation durations=62.78 to 56.09 days, Fig. 5B) as well as for proxy 8 (P=21.31 days, S=0.018, Transitional Range of TSP durations=22.66 to 20.07 days, Fig. 5C) (Table 2).

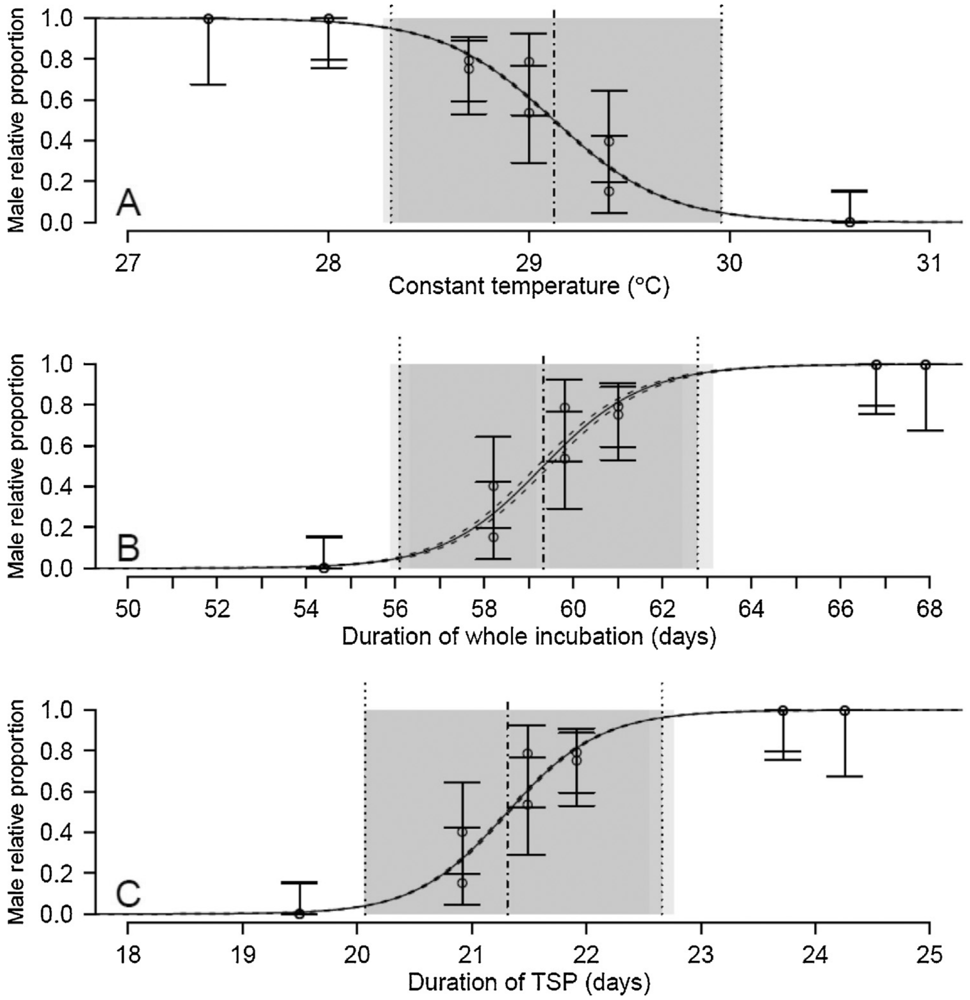

Fig. 5. Patterns of temperature-dependent sex determination (TSD). Relationship between true sex ratio (black points are the average sex ratio and error bars show the confidence interval) from laboratory experiments and constant incubation temperature (A), the whole incubation period (B), and the duration of the thermosensitive period (TSP) (C) based on constant temperature experiments (Marcovaldi et al., 1997) The Transitional Range of Temperatures (TRT) is indicated by the grey area in between the horizontal dotted lines. Shades of grey indicate the confidence interval of the boundaries of the TRT at 5%.

3.4. Sex ratio from temperature-based proxies (proxies 1–6)

Sex ratio was predicted for each of the 38 natural nests (Appendix B in Supplementary material). Sex ratio of nests laid after the end of November was estimated to be 100% females regardless of the proxy used (Fig. 6). For nests laid before the end of November, sex ratio estimates differed if either CTE_A or CTE_W were used and for the different incubation periods considered. The average sex ratio for all clutches and the dispersion of sex ratio among clutches (lower and upper quantiles at 2.5%) were estimated. On average, more females were estimated if sex ratio was based on CTE_A or CTE_W during the whole incubation period (up to 90% females) rather than CTE_A or CTE_W during the middle third of incubation or the TSP (less than 60% females based on CTE_A and less than 80% females based on CTE_W, Fig. 6, respectively A and B). Importantly, the dispersion of sex ratio among nests is similar when estimated from CTE_A or CTE_W during the middle third of incubation or during the TSP but differs considerably when predicted from either CTE_A or CTE_W during the whole incubation period. During October, sex ratio of clutches estimated from CTE_A during either the middle third of incubation or the TSP shows similar dispersion (10% to 80% females and 20% to 90% females, respectively for the middle third of incubation and the TSP) whereas it ranges from 60% to 100% females when based on CTE_A during the whole incubation (Fig. 6A). Similarly, sex ratio ranged from 20% to 95% females when based on CTE_W during either the middle third of incubation or the TSP whereas it ranges from 80% to 100% females when estimated from CTE_W during the whole incubation (Fig. 6B).

The overall difference in sex ratio estimates among the three developmental periods is less pronounced if CTE_W is used rather than CTE_A (Table 3).

3.5. Sex ratio from duration-based proxies (proxies 7 and 8)

Whole incubation durations of natural nests ranged from 46.5 to 63.5 days (mean = 52.9 days, sd = 4.2) with a slight decreasing trend along the nesting season (Appendix A in Supplementary material). Average sex ratio during the last 15 days of October differs substantially if whole incubation durations are used as a proxy rather than TSP durations (60% and 35% females respectively for whole incubation durations and TSP durations). After mid-November, on average 100% females average is expected regardless of the proxy used (Fig. 6C). Only one nest was monitored during the first half of February so this difference cannot be discussed. Moreover, the dispersion of sex ratio expected during 15-days periods differed according to the proxy. The dispersion of sex ratio expected from TSP durations is always narrower than expected from whole incubation durations. This is particularly the case during the last 15 days

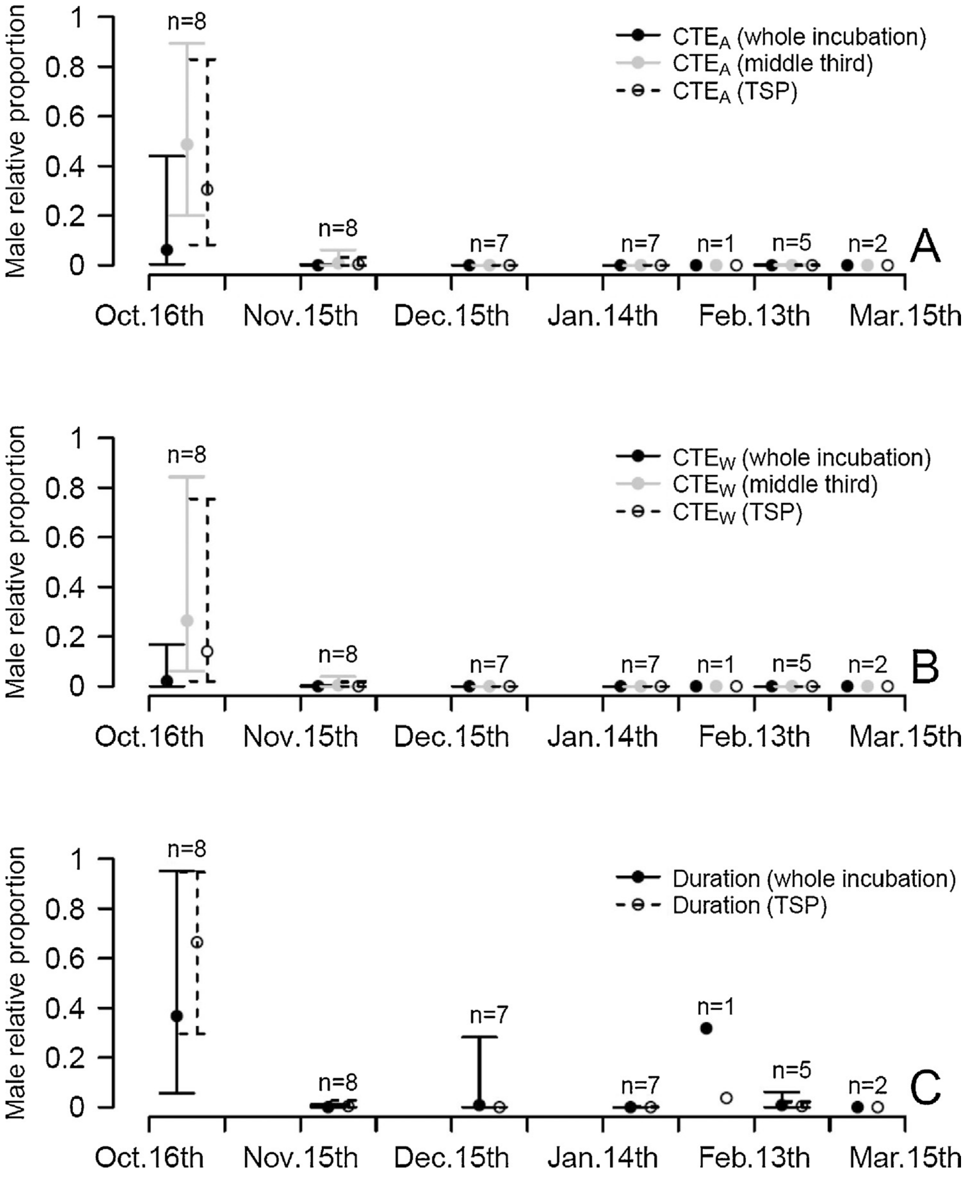

Fig. 6. Sex ratio predicted for natural nests incubated at Praia do Forte during the 2014–2015 nesting season. Estimates are based on CTEA (A), CTEW (B) and durations of developmental periods (C). Error bars show lower (2.5%) and upper (97.5%) quantiles for sex ratio estimated for each 15-days period (n indicates the number of nest that were laid within the 15-days periods).

of December when 100% females were predicted from TSP durations whereas 70% to 100% females were predicted from whole incubation durations. The averaged sex ratio for the 38 clutches is expected to be 90% females when whole incubation durations are used as proxies and 86.2% when TSP durations are used. The overall dispersion of sex ratio is expected to range from approximately 100% to 13% females when whole incubation durations are used as compared to 100% to 8.4% when TSP durations are used (Table 3).

3.6. Quantifying differences among methods

Euclidean distance for each pairwise comparison of proxy-specific sex ratio estimates have been calculated. The highest distance is observed for comparison between sex ratio estimates based on CTE$_W$ during the whole incubation and based on the duration of the TSP. The lowest distance is observed for comparison between sex ratio estimates based on CTE$_A$ during the TSP and CTE$_W$ during the TSP (Table 4).

The dendrogram shows two main branches: one with two leaves that correspond to sex ratio estimates based on duration-based proxies (duration of the whole incubation and duration of the TSP) and another one with six leaves that correspond to sex ratio estimates based on temperature-based proxies (CTE$_A$ and CTE$_W$ during the whole incubation, during the middle third of incubation and during the TSP) (Fig. 7). Two sub-groups can be identified within the branch of temperature-based proxies for sex ratio: one with two leaves which corresponds to CTE$_A$ and CTE$_W$ during the whole incubation period and another one with four leaves that corresponds to CTE$_A$ and CTE$_W$ during the middle third of incubation and during the TSP. The structure of the dendrogram is well supported by data (approximately unbiased bootstrap values between 79% and 100%, Fig. 7).

4. Discussion

Sex ratio estimates differ greatly depending on the proxy being used. A higher proportion of females tend to be estimated for temperature based proxies (CTE$_A$ or CTE$_W$) when the whole incubation period was used rather than the middle third of incubation or the thermosensitive period for sex determination (TSP). For duration

Table 3
Average and quantiles for sex ratio predicted for the 38 natural nests according to each proxy.

Proxy	Mean sex ratio (% of males)	Lower quantile (2.5%)	Upper quantile (97.5%)
CTE$_A$ (whole incubation)	2.05%	<0.1%	16.40%
CTE$_A$ (middle third of incubation)	10.40%	<0.1%	58.90%
CTE$_A$ (middle-third of development)	6.84%	<0.1%	45.40%
CTE$_w$ (whole incubation)	0.79%	<0.1%	8.92%
CTE$_w$ (middle third of incubation)	5.99%	<0.1%	37.00%
CTE$_w$ (middle-third of development)	3.86%	<0.1%	28.10%
Duration-based equivalent in days(whole incubation)	9.97%	<0.1%	86.20%
Duration-based equivalent in days (Middle-third of development)	13.80%	<0.1%	91.60%

Table 4
Matrix Euclidean distances of pairwise comparison of each proxy-specific sex ratio estimates.

	CTE$_A$ (whole incubation)	CTE$_A$ (middle third of incubation)	CTE$_A$ (middle-third of development)	CTE$_w$ (middle third of incubation)	CTE$_w$ (middle third of incubation)	CTE$_w$ (middle-third of development)	Duration-based equivalent in days (whole incubation)
CTE$_A$ (middle third of incubation)	1.13						
CTE$_A$ (middle-third of development)	0.69	0.50					
CTE$_w$ (whole incubation)	0.32	1.33	0.96				
CTE$_w$ (middle third of incubation)	0.61	0.64	0.21	0.90			
CTE$_w$ (middle-third of development)	0.39	0.91	0.42	0.71	0.29		
Duration-based equivalent in days (whole incubation)	1.31	1.29	1.18	1.40	1.25	1.29	
Duration-based equivalent in days (Middle-third of development)	1.64	0.94	1.17	1.80	1.28	1.48	0.96

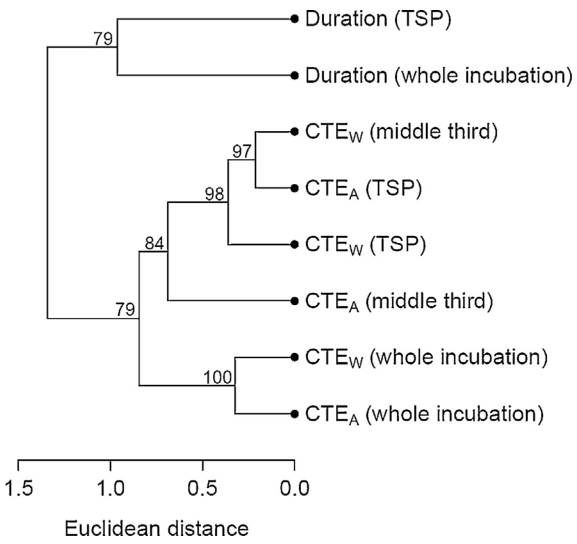

Fig. 7. Clustering of sex ratio estimates. Dendrogram based on Euclidean distances of each proxy-specific sex ratio estimates. Approximated unbiased bootstrap values (in percentage) are indicated at nodes.

based proxies the whole incubation duration produced a wider range of sex ratio than the TSP durations, being longer, incubation duration are more variable than TSP durations and thus estimate a wider range of sex ratio. Further, temperature experienced during the TSP is more similar among nests whereas temperature experienced out of TSP boundaries may be much more different among nests. Ideally we would compare predictions to the true sex ratio of nests, however for this the sacrifice of hatchlings is needed. Thus, CTE$_W$ during the TSP was used as a reference to discussing differences among methods since it is the closest proxy to the marine turtle mechanism of TSD (Georges et al., 1994; Georges et al., 2005). When compared to estimates from this proxy, the CTE$_A$ during the TSP and the CTE$_W$ during the middle third of incubation gave similar sex ratio estimates whereas estimates using the CTE$_W$ and CTE$_A$ during the whole incubation period or both duration based proxies were substantially different. Estimates using CTE$_A$ during the middle third of incubation also provided unsuitable discrepancy.

4.1. Temporal scale of proxies

Marine turtle sex ratio is determined only during the TSP which corresponds to the middle third of development (Yntema and Mrosovsky, 1982; Georges et al., 2005; The Chu et al., 2008; Woolgar et al., 2013). Thus, calculating sex ratio with temperatures from the whole incubation period is inaccurate. In an attempt to provide more accurate estimates, the middle third of development has been widely used as the middle third of the whole incubation period (Sieg et al., 2011; Maulany et al., 2012; Gomuttapong et al., 2013; Jribi et al., 2013b; King et al., 2013; Jribi and Bradai 2014; Candan and Kolankaya 2016; Önder and Candan 2016; Yalçli Özdilek et al., 2016). However, this is true only when temperature remains constant during incubation. If temperature fluctuates the boundaries of the true TSP during incubation period does not necessarily fall within the middle third of the whole incubation period (The Chu et al., 2008). In nature, various climatic events have an incidence on the temperature within the nest. Temperature can vary stochastically due to precipitations, wind speed or cloud cover and deterministically due to daily and seasonally solar cycles. The extent to which nest temperature is affected by those meteorological and climatic parameters depends on thermal properties of the incubation substratum and the depth at which the nest is buried. Even when marine turtles bury their eggs deep enough to buffer

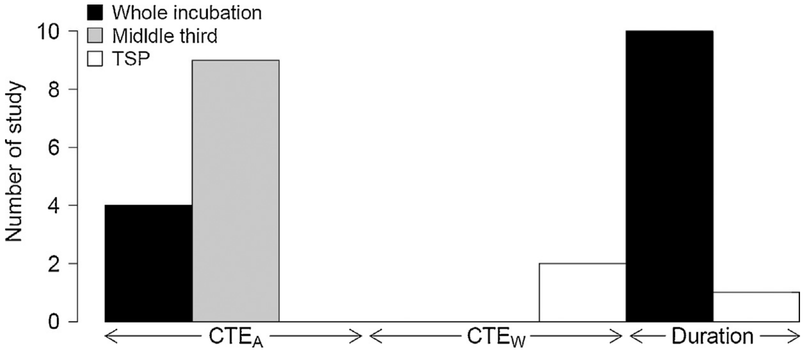

Fig. 8. Proxies used in the literature to infer sex ratio. Number of studies that have used each proxy to predict hatchling sex ratio of sea turtles from recorded traces of temperature between 2010 and 2016 (Steckenreuter et al., 2010; Sieg et al., 2011; Katselidis et al., 2012; LeBlanc et al., 2012a; LeBlanc et al., 2012b; Maulany et al., 2012; Fuller et al., 2013; Gomuttapong et al., 2013; Jribi et al., 2013a; King et al., 2013; dei Marcovaldi et al., 2014; Jribi and Bradai 2014; Kılıç and Candan 2014; Simões et al., 2014; Stubbs et al., 2014; Rocha et al., 2015; Sarı and Kaska 2015; Tapilatu and Ballamu 2015; Candan and Kolankaya 2016; dei Marcovaldi et al., 2016; Yalçli Özdilek et al., 2016).

temperature fluctuations, a slight temperature increase or decrease during incubation invalidates methods that approximate the true TSP by the middle third of the whole incubation period. Therefore, it is crucial to determine the moment at which each developmental stages occurs to accurately determine the true TSP boundaries. Monitoring the progression of embryo growth may require the sacrifice of embryos, however alternative methods are now available (Georges et al., 2005; Girondot and Kaska 2014).

4.2. Using thermal reaction norm for growth rate

Caution should also be made to not oversimplify the effect of temperature on sex determination by estimating sex ratio based on the average temperature (CTE$_A$) during any developmental periods. This make the wrong assumption that embryonic growth rate has no impact on sex determination (Georges 1989; Georges et al., 1994; Georges et al., 2005; Neuwald and Valenzuela 2011). If an embryo of a species with MF TSD pattern is exposed 12 h at male-producing temperatures and 12 h at female-producing temperatures, then few cells will proliferate under male-producing temperature and a larger amount at female-producing temperature. As an alternative, it is suggested to estimate the sex ratio based on the incubation temperature at which half of the development has occurred (developmental median) (Georges 1989; Georges et al., 1994; Georges et al., 2005). This requires to accessing or modelling embryo morphometric. As another alternative, it has been proposed that the duration of the whole incubation can be used as a proxy for sex ratio (Godfrey 1997). This proxy is not invasive and easily measurable in the field. This assumes that the thermal reaction norm for the growth rate of embryos is monotonic within the thermal range experienced by embryos during incubation. Which means that the higher the temperature, the shorter the incubation and, in turn, more feminizing. However, a wide diversity of sex ratio can be observed for the same incubation duration when temperature fluctuates (Georges et al., 1994). Moreover, this method does not enable to get the duration of the TSP. The thermal reaction norm for growth rate of loggerhead population nesting at Praia do Forte is not monotonic with an optimum at 34.5 °C. This is consistent with previous observation that turtle embryos incubated at 35 °C or higher temperature; it may still develop but suffer of developmental abnormalities or even not develop successfully (Pieau 1978; Valverde et al., 2010). When the thermal reaction for growth rate is non- monotonic within the range of incubation temperature embryos can exhibit the same growth rate for two different temperatures. This has been observed for loggerhead populations in Turkey (Girondot and Kaska 2014) and Australia (Woolgar et al., 2013). It is therefore possible that embryos that have incubated in distinct thermal environment could present the same TSP duration. In such cases, the CTE$_W$ during the TSP is the only reliable proxy to predict sex ratio of TSD species.

4.3. Proxies used to infer sex ratio

It is acknowledged that the mean nest temperature is a poor proxy to predict sex ratio of TSD species under natural fluctuating thermal regimes because more development would occur above the mean than below (Bull and Vogt 1981; Pieau 1982; Mrosovsky et al., 1984; Bull 1985; Georges 1989; Georges et al., 1994; Valenzuela 2001; Georges et al., 2005,; Neuwald and Valenzuela 2011) and that the TSP lies in the middle third of development (Yntema 1979; Bull and Vogt 1981). The first type of CTE$_W$ have been formulated by Georges (1989) and have been implemented by some studies, which acknowledges that the true TSP does not necessarily lie during the middle third of incubation in natural conditions (Valenzuela 2001; Georges et al., 2005; The Chu et al., 2008; Neuwald and Valenzuela 2011; Woolgar et al., 2013; Stubbs et al., 2014). At least three models have been proposed to account for thermal reaction norm for the rate of development while calculating a proxy to predict sex ratio: (i) degree.hours, (ii) Dallwitz-Higgins and (iii) Sharpe-DeMichele (Georges et al., 2005). The degree.hours model assumes that the developmental rate is linear and monotonic within any thermal range which is not relevant for most situations. However, Dallwitz-Higgins and Sharpe-DeMichele models provide two different equations that are able to account for a decrease in developmental rates at high temperatures. These models are very similar (Georges et al., 2005) and have already been used to calculate CTE$_W$ (Stubbs et al., 2014; Tapilatu and Ballamu 2015). Despite an improvement in predicting the sex ratio of TSD species, the most used proxies to predict sex ratio of marine turtles from traces of temperature under natural condition for the last decade (2010–2016) are still (i) the duration of the whole incubation period (ii) the CTE$_A$ during the middle third of the whole incubation period (simple average) and (iii) the CTE$_A$ during the whole incubation period (Fig. 8).

This is a reflection of the ease to collect information on the incubation duration of nests. Although, the duration of the whole incubation does allow for a rapid assessment of incubation conditions, it must be stressed out that sex ratio estimation based on this proxy is not reliable. However it can give information about nests incubated at higher temperatures and at risk of reaching temperatures at the upper thermal threshold, which can help trigger active responses. Importantly, when selecting the best proxy to use, consideration needs to be undertaken both on the reliability of sex ratio estimate and on the logistical and feasible opportunities for data collection at each site.

4.4. Using proxies to predict future sex ratios

A common goal of studies assessing marine turtle viability under climate change conditions is to predict future sex ratio under different climate scenarios. However, most studies use oversimplified proxies, such as monthly mean temperature as a proxy of nest temperature and consider the middle third of the whole incubation period as the TSP for sex determination, affecting the reliability of these studies. Importantly, we showed that nest temperature time series exhibit specific statistical properties (autoregressive process) that should be taken into account when predicting sex ratio. Consideration should also be given to the fact that other temperature-dependent traits are interacting at the same time. For example, survival rate of embryos before hatching depends on incubation temperature (Howard et al., 2014) and the nesting dynamic of adult females may depend on the sea surface temperature at the feeding and breeding sites through resources availability and energy expenditure (Weishampel et al., 2004; Pike et al., 2006; Mazaris et al., 2009; Weishampel et al., 2010; Ben Hassine et al., 2011). Together, these parameters can amplify, buffer or even neutralize the effect of climate change on embryonic development of marine turtles, altering the sex ratio being produced. Importantly, further studies are needed to explore the sensitivity of embryos to extreme temperatures. Thus, information on temperature-dependent hatching success is required to provide reliable primary sex ratio estimates and thus assess the vulnerability of TSD species under different climate change conditions.

5. Conclusion

Several proxies are used to estimate the sex ratio of marine turtle hatchlings. Here we showed that sex ratio estimates differ greatly according to the proxy used. CTE_W during the TSP is likely the most relevant biological proxy thus likely produces the most reliable sex ratio estimates. In natural conditions, temperature can vary either stochastically or seasonally. Given this, growth rate of embryos is not expected to be constant. Hence, the thermosensitive period for sex determination can start earlier or later and last shorter or longer depending on environmental thermal variations. Thus, the need to adequately determine the TSP and to obtain appropriate estimates of embryo growth rate and the influences of temperature. Ultimately, further information should be obtained on how to model temperature-dependent hatching success in natural conditions in order to improve our understanding of the impact of climate change on the developmental stage of TSD species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ecolmodel.2017.09.022.

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