*Research Article*

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# Modelling the Sex Ratio of Natural Clutches of the European Pond Turtle, *Emys orbicularis* (L., 1758), from Air Temperature

***Jonathan Monsinjon, Jean-Michel Guillon, Vincent Hulin & Marc Girondot****

Laboratoire Ecologie, Systématique et Evolution, Université Paris Sud, CNRS, AgroParisTech, Université Paris Saclay, 91405 Orsay cedex, France

**Abstract:** Temperature-dependent sex determination (TSD) is a kind of sex determination in which gonadal sex is dependent on the incubation temperature of the eggs. Within reptiles, TSD occurs in many turtles, all crocodilians, the tuataras and some lizards. The European Pond Turtle, *Emys orbicularis*, has been the most studied species for TSD, and the biochemistry of TSD is well-understood in this species. However, predicting the sex ratio when incubation temperature is not constant remains challenging. Here we propose a new methodology to predict the sex ratio for natural nests, and we apply this methodology to eggs of *E. orbicularis* incubated under natural conditions. Finally, we give recommendations on how to apply this method to other natural populations or species with TSD.

**Key words**: air temperature, soil temperature, nest temperature, thermosensitive period of sex determination, embryonic development, temperature-dependent sex determination

## Introduction

Two sex determination mechanisms are found in reptiles: genotypic sex determination (GSD) where the sex is predetermined during egg fertilization by the genotype, and temperature-dependent sex determination (TSD) where the temperature of the embryo during incubation defines the sex (Pieau 1996). In reptiles, TSD was first demonstrated for the lizard *Agama agama* (Charnier 1966), and a few years later for the turtles *Testudo graeca* and *Emys orbicularis* (Pieau 1971, 1972). The latter has been the model species for the study of TSD by Claude Pieau for 35 years and is so far the most studied reptile for TSD (Pieau & Dorizzi 2004). However, only individuals from the center of France have been studied for TSD, though the species ranges from Portugal (Sillero et al. 2014) to Kazakhstan (Mazanaeva & Orlova 2004).

Animals with temperature dependent sexual determination are susceptible to experience a sex ratio bias depending on incubation conditions. The sex ratio bias could be still enhanced due to climate change. Sex ratio bias can have profound consequences in term of population dynamics and then for conservation because it can promote failure in fertilization rate if one sex is depleted, over-exploitation of resources if males and females do not use the same resources in the environment, and loss of genetic variation impacting the viability of populations due to lower effective size of population. This lowering of genetic variation could jeopardize the ability of turtle populations to adapt to climate change and then drive the population to an extinction vortex.

The European Pond Turtle, *E. orbicularis*, shows the Males-Females (MF) pattern of TSD typical for many turtles: incubation temperatures lower than 27°C are masculinizing, while temperatures higher than 30°C are feminizing (Girondot 1999). The range of temperatures (28.51±1.15°C for *E. orbicularis*, see Girondot 1999) producing both sexes is the transitional range of temperatures (TRT);

*Corresponding author: marc.girondot@u-psud.fr

the temperature producing both sexes in equal proportion is the pivotal temperature (PT) (Mrosovsky & Pieau 1991). Usually, TRT and PT are identified for incubations at constant temperatures. In nature, where the temperature often changes during development, these statistics do not predict the sex ratio correctly (reviewed in Hulin et al. 2008).

However, the embryo temperature is important for the sex determination only during the thermosensitive period (TSP), or the period of the development during which temperature exerts its influence on the sexual phenotype of the gonad (Mrosovsky & Pieau 1991). The TSP in *E. orbicularis* is between stages 16 (when the gonads appear) and 21, when embryos have mass 160–180 mg and 650–750 mg, respectively (Pieau & Dorizzi 1981). When incubation temperature is constant, this range of masses is observed during the middle third of incubation. However, this correspondence is not true when temperature changes during incubation according to daily fluctuations and seasonal trends. In natural nests, embryos experience a fluctuating regime of temperatures. TSD has been mostly studied under laboratory conditions at constant incubation temperatures, and consequently the effects of fluctuating temperatures during incubation are understood much less. Thus in order to correctly predict the timing of temperature effect for sex determination during incubation, it is necessary to stage the embryo. Staging the embryo requires to open the egg which cannot be done for threatened species. An alternative is to model the embryo development to determine the boundaries of the TSP during incubation and then predict with better accuracy the sex ratio.

In this paper, we present the successive steps for predicting sex ratio under natural conditions using the European Pond Turtle, *E. orbicularis*, as a target species but the methodology can be applied to any species with TSD. We start with obtaining air or soil temperatures recorded by meteorological stations in the vicinity of nesting sites, and we describe how to predict nest temperatures. Then, we show how nest temperatures may be used for determining the TSP, and how the TSP duration or temperatures, in turn, can be used to estimate nest sex ratio.

# Methodology

**Step 1: Obtaining air and soil temperatures**

Besides planting automated dataloggers (e.g. iButtons, Hobo) in the field, often data from meteorological stations are (freely) available and can provide a convenient starting point. Guidelines for meteorological stations deployments are used worldwide to ensure that comparable measurements are obtained (World Meteorological Organization 2008). Measurements can represent highest, lowest, and mean air temperatures, and ground temperature or soil temperatures at various depths. General recommendation for meteorological stations indicate that measurement equipment must be located in the middle of a flat area of 25 × 25 m without any trees or building in proximity that could cast shade. The ground should be covered only by short grass or the local typical soil cover. The observed air temperature should be representative of the free air conditions surrounding the station. Temperature must be recorded at a height of 1.2–2.0 m above ground level. To ensure that the thermometer is at true air temperature, protect it from radiation by a screen. For all measurements, each thermometer must be compared with a reference thermometer, or thermometers of the same type can be compared with each other. This calibration should also be done when data loggers are used during fieldwork to ensure that differences between sites do not reflect differences between thermometer calibrations. If data are collected using personal dataloggers, similar considerations should be taken into account to have reproducible and comparable temperature measurements.

The daily air temperature typically shows an irregular sinusoidal pattern with a 24-hours period (the pattern is irregular because the time for maximum is not at the exact intermediate between the two times for the minima). For a particular day this pattern can be degraded by local meteorological events, but here we only discuss the average pattern, which is representative of the local climate. To illustrate the pattern of daily temperatures, we used air temperature recorded by the laboratory Ecologie, Systématique, Evolution each 30 minutes from 2006 to 2014 in Barbeau, ~32 km south-east of Paris in France (N43°03’07.801’’ E22°56’34.148’’). Time was converted to Universal Time Coordinated (UTC) to prevent hour shift between summer- and wintertime. The time of sunrise and sunset were obtained for each day at this location based on classical formula (Teets 2003). It is important to use the local time for sunrise or sunset because time can be different by more than one hour as compared to sun time between the two extreme longitudes of a time zone. For each day, the UTC times of the daily minimum and maximum temperatures were calculated based on the time series of temperatures. The mode for the minimum and maximum temperature time has been estimated according to UTC time of sunrise grouped by 30 minutes using the method of Asselin de Beauville (1978). Minimum temperature is approximately 30 minutes after sunrise. Maximum

temperature is generally between 13:00 and 15:00 h depending on the latitude and the time of sunrise, thus the season (Fig. 1). There is little chance that the true minimum and maximum are exactly represented in time series when temperatures are recorded every couple of hours, so the true daily minimum and maximum must be estimated using fit of irregular sinusoidal pattern (function minmax.periodic () in the HelpersMG R package; see Girondot 2016b).

The standard depths for soil temperature measurements in meteorological station are 5, 10, 20, 50 and 100 cm below the surface. As a standard procedure the soil type, soil cover and the degree and direction of the ground's slope should be recorded. Whenever possible, the physical soil constants, such as bulk density, thermal conductivity, the level of the water table (if within 5 m of the surface) and the moisture content at field capacity should also be indicated. The soil temperature follows a similar daily pattern as the air temperature, but with some time shift depending on depth, amplitude change, and soil heat conductivity. Soil heat conductivity is itself dependent on the granular constitution of the soil, its composition and its humidity (Abu-Hamdeh & Reederb 2000). The physics of heat transfer into heterogeneous media such as soil is complicated and correlative studies are often the only way to estimate heat conductivity (Farouki 1981).

**Step 2: From air or soil temperatures to nest temperatures**

Like soil temperatures, temperature within the nest depends on the air temperature, sun irradiation but also soil heat conductivity. At the depth of a typical nest of *E. orbicularis* (around 10 cm), the time shift with air temperature is very small, around 20 minutes (Guyot 2013) and the nest temperature is correlated with the soil temperature but their averages and amplitudes can be different. As an example, temperatures have been recorded in nine *E. orbicularis* nests in Brenne, 200 km south of Paris, in July and August 2009 every 3 hours; corresponding temperatures for air at 2 m and soil between 7 to 26 cm have been obtained from ECMWF project every 6 hours at a nearby station (Fig. 2).

Two methods can be used to predict the nest temperature from the soil or air temperatures obtained

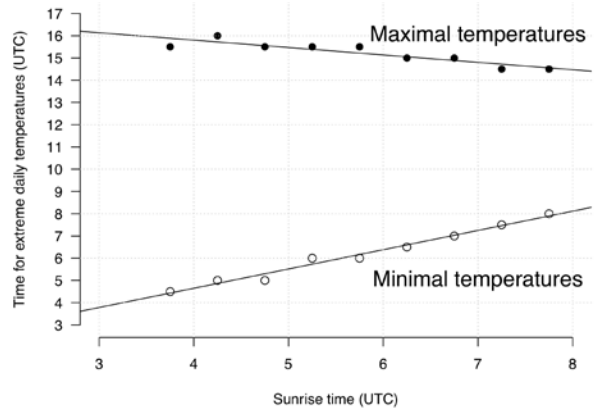


**Fig. 1.** Time for minimum and maximum daily air temperature from 2006 to 2014 at Barbeau in France (N43°03'07.801" E22°56'34.148") according to the time of sunrise

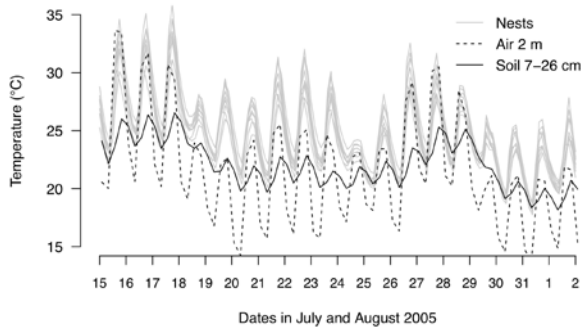


**Fig. 2.** *Emys orbicularis* nest temperature recorded in nine nests in 2005 in Migné, France (N46°42'36" E01°18'36"), air temperature at 2 m high and soil temperature between 7 to 26 cm depths from ECMWF project (N46°36'00" E01°15'00")

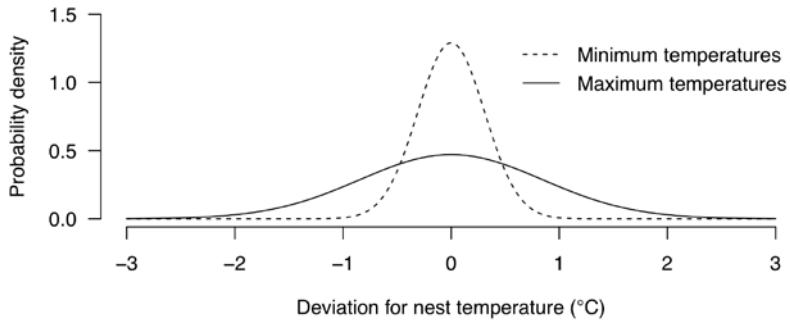


**Fig. 3.** Distribution of random factor in generalized linear mixed model (GLMM) measuring the heterogeneity of different location for daily minimum and maximum temperatures, estimated from nine nests of *Emys orbicularis* monitored in 2005

by meteorological stations. A physical model of heat transfer can be defined (Farouki 1981), but many physical constants must be determined and such data are often not available. A more practical solution is to calibrate a model relating nest temperature obtained locally with data loggers to air or soil temperatures obtained from meteorological stations or from data loggers close to the nesting area. Rather than fitting average temperature and amplitude, we find it more suitable to fit minimum and maximum daily temperatures separately. A generalized linear mixed model (Gaussian error and identity link) relating the nest temperature to the air or soil temperatures for the minimum and maximum temperatures must be built separately for calibration. The maximum daily temperature is from 0:00 h of day i to 0:00 h of day i+1 and the minimum daily temperature is from 12:00 h of day i to 12:00 h of day i+1. This shift permits the fit of some cases when the minimum temperature could be before midnight due to local meteorological conditions (Eccel 2010). Thus, we obtain the following models, where i indicates the day, $\varepsilon_i$ is the Gaussian error term for the day i, and $\mu_L$ is the random effect dependent on the logger L:

$T_{max}$ Nest (0:00 h → 0:00 h)$_i$ ~ $T_{max}$ Air or Soil (0 h → 0 h)$_i$ + $\mu_L$ + $\varepsilon_i$

$T_{min}$ Nest (12:00 h → 12:00 h)$_i$ ~ $T_{min}$ Air or Soil (12 h → 12 h)$_i$ + $\mu_L$ + $\varepsilon_i$

The construction of these linear models must include autocorrelation and moving average within the error structure (ARIMA model) and the data loggers must be used as a random factor to reflect their different localization. The air or soil temperatures are treated as fixed effects. The output for fixed factors provides estimates for mean-differences or slopes. Random factors, on the other hand, are defined by a distribution and not by differences. The output for a random factor is an estimate of this variance and not a set of differences from a mean (Pinheiro & Bates 2000). Distribution of the random factor can be used as a measure of heterogeneity of nest temperatures as compared to air or soil temperature. For the nine nests monitored in Brenne (Fig. 2), the distributions of logger random factors for minimum and maximum temperatures are shown in Fig. 3: heterogeneity of temperatures is higher for maximal temperatures than for minimal temperatures. Sometimes, the temperature difference can be easily explained by the amount of vegetation cover or tree shade, but sometimes the locations look similar and the difference is probably the consequence of difference in soil conductivity due to difference of soil composition and humidity. Using these statistical tools, it is possible to calibrate nest temperatures based on air or soil temperatures recorded from meteorological stations, integrating thermal heterogeneity. This calibration can then be used to predict nest temperatures in a nesting area from a local pattern of air or soil temperature variation.

**Step 3: From nest temperature to thermosensitive-period of development for sex determination**

A model of embryo growth can be used to estimate the exact location of the TSP during development for a nest incubated in natural conditions. Determining the thermal reaction norm for *E. orbicularis'* embryonic growth rate was necessary to build such a model. A reaction norm is the relationship between a phenotypic character and an environmental factor. Here the environmental factor is the incubation temperature and the phenotypic character is the embryonic growth rate. When the thermal reaction norm for embryo growth is known, it is possible to model the embryo mass or straight carapace length (SCL) change for any time series of nest temperatures. Such

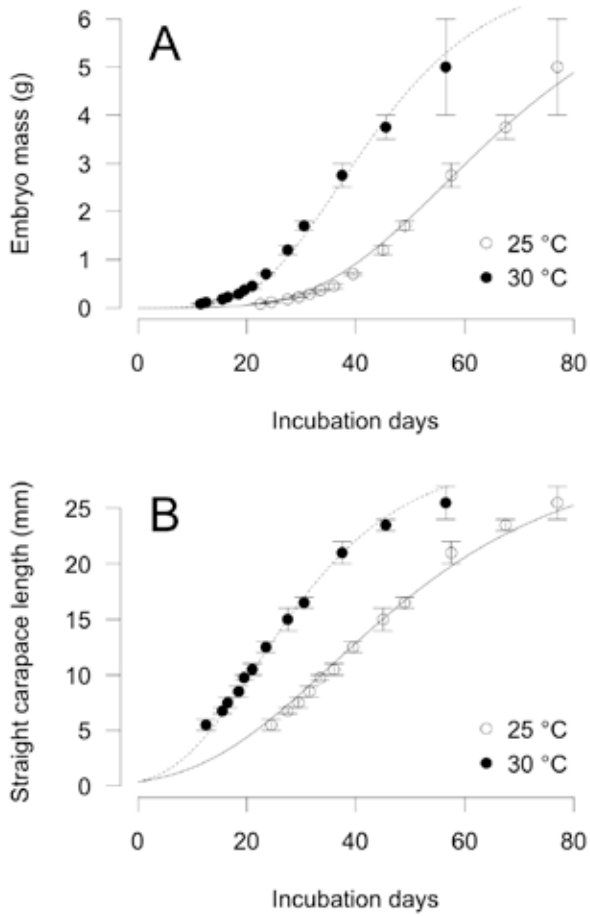


**Fig. 4.** (A) Embryo mass and (B) straight carapace length (SCL) during development at two constant incubation temperatures (points and error bars being 2×SD) and fit of Gompertz growth model at both temperatures with a common size at day 0 ($X_0$ parameter) and $K$ parameter

a model has been published recently for the marine turtle *Caretta caretta* (Girondot & Kaska 2014) and has been converted here for *E. orbicularis*. Information of quantitative development for eggs incubated at constant temperatures (Fig. 4) and from two nests incubated at non-constant temperatures (Fig. 5) has been used to model the thermal reaction norm for embryo growth (Fig. 6). We choose to model the change in straight carapace length (SCL) rather than mass because SCL is strictly increasing during development whereas mass can decrease due to loss of water in dry substrate or at high incubation temperature. The thermosensitive period during embryo growth was determined using a table for correspondence between mass or SCL and embryological stage (Pieau & Dorizzi 1981). It is clear that the TSP is not located at the middle third of incubation when temperature is not constant (Fig. 5).

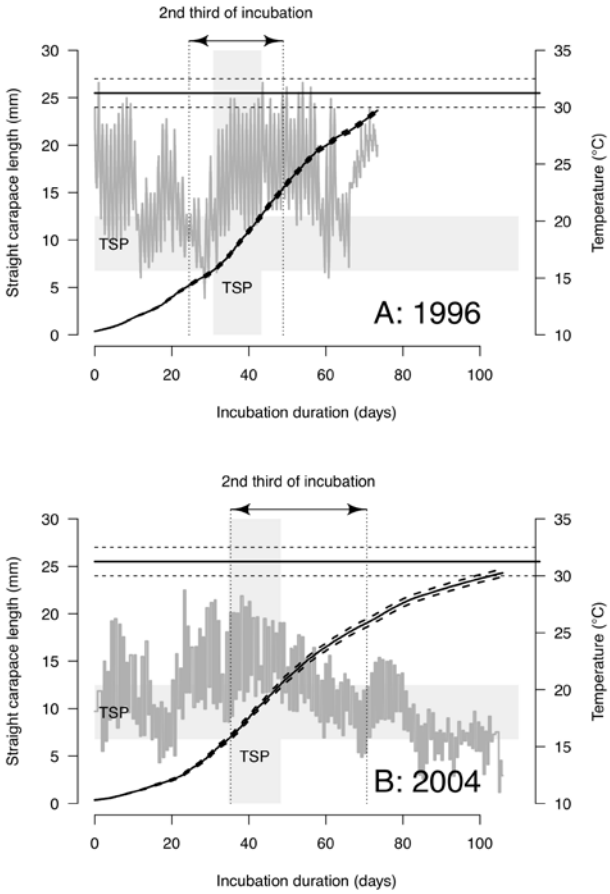


**Fig. 5.** Modeled *Emys orbicularis* embryonic straight carapace length (SCL) growth incubated *in situ* in (A) 1996 and (B) 2004. Grey lines are the temperatures within the nest and the black lines represent the model for the growth of the embryo. Horizontal lines indicate the observed Straight Carapace Length (SCL) of *E. orbicularis* hatchlings and its 95% confidence interval. Grey rectangle is the position of thermosensitive-period for sex determination according to embryo SCL on SCL-axis (Pieau & Dorizzi 1981) and its projection on time-axis. Vertical dot lines and the corresponding arrows at the top delineate the second-third of incubation

**Step 4: Constant temperature incubations and sex ratio**

A total of 1,259 eggs of *E. orbicularis* have been incubated at constant temperatures and sexed for Claude Pieau' researches from 1970 to 1998 (reviewed by Girondot 1999). The relationship between sex ratio and incubation temperature can be fitted using different sigmoidal functions (Girondot 1999, Godfrey et al. 2003, Hulin et al. 2009). We introduce here a new one that we name double-Richards. It is similar to the Richards equation (see Godfrey et al. 2003) with two $K$ (parameter for asymmetry) values ($K_1$ and $K_2$) below and above the PT. Among these models only the logistic model is symmetrical. An asymmetrical model (Hulin) is selected based on Akaike information criterion and Akaike weight

**Table 1.** Fit of sex ratio depending on constant incubation temperatures for 1,259 eggs (reviewed in Girondot 1999). Hulin model is selected based on AIC and Akaike weight (shown graphically in Fig. 6). AIC is the Akaike Information Criterion which is a measure of the fit of the model corrected for over-parametrization, ΔAIC is the difference with the lowest AIC, and Akaike weight is the probability that a model is really the best (Burnham & Anderson 2002)

| | Parameters | AIC | ΔAIC | Akaike weight |
|---|---|---|---|---|
| Logistic | 2 | 93.44 | 9.22 | 0.008 |
| Hill | 2 | 93.14 | 8.92 | 0.01 |
| Richards | 3 | 88.40 | 4.18 | 0.10 |
| Hulin | 4 | 84.22 | 0.00 | 0.83 |
| Double-Richards | 4 | 90.09 | 5.87 | 0.04 |

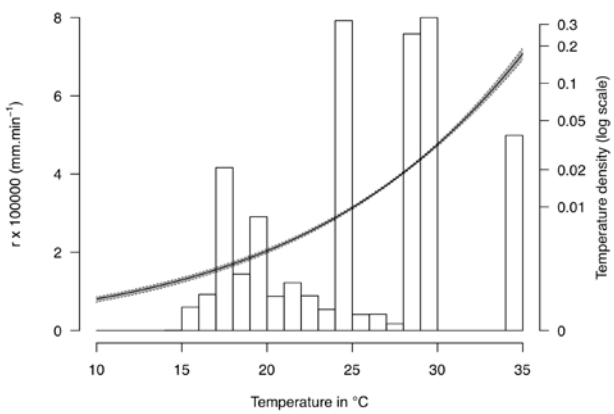


**Fig. 6.** Embryo growth thermal reaction norm (line) and the histogram of temperatures in two nests used for fitting

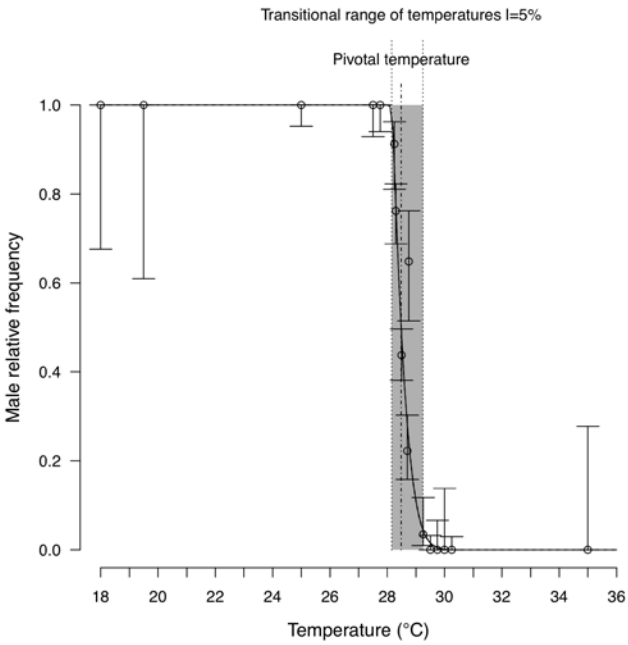


**Fig. 7.** Sex ratio (proportion of males) of *Emys orbicularis* embryos from Brenne (France) incubated at constant temperatures. The error bars represent twice the standard deviation for each point estimate. The solid line shows the selected model based on table 1. The pivotal temperature is 28.48°C (standard error <0.001) and the transitional range of temperatures (shaded area) defined as the range of temperatures producing sex ratio between *l* and 1-*l* (*l*=5%) is 1.05°C (standard error <0.001) [28.16°C–29.21°C]

(Table 1). Thus, we identified *P*=28.48°C as the PT [CI 95%=(28.481, 28.482)] (Fig. 7), with the sex ratios at *P*-0.3°C and *P*+0.3°C being 0.95=0.5+0.45 and 0.21=0.5-0.19, respectively. In other words, the feminizing effect of temperature is not symmetrical around the PT, which means that it is necessary to add more thermal energy to feminize when the temperature increases below PT rather than above.

**Step 5: From thermosensitive-period of development for sex determination to sex ratio**

A common mistake is to use the average temperature recorded during incubation to predict sex ratio. This has been elegantly demonstrated for the marine turtle *C. caretta* when eggs were incubated at the same average temperature but with different daily amplitude: the larger the amplitudes, the more feminized the embryos (Georges 1989, Georges et al. 1994). To understand the effect of changing temperatures on sex ratio, imagine that you could freeze embryos and that they will survive. Freezing temperatures should be masculinizing for a MF TSD pattern. But the embryos do not develop at all and, as a direct consequence, this low temperature does not impact their metabolism and will have no effect for

**Table 2.** Fit of sex ratio upon duration of the thermosensitive period of development for sex determination. Hulin model is selected based on AIC and Akaike weight (shown graphically in Fig. 8). See table 1 for the legend of columns

| | Parameters | AIC | ΔAIC | Akaike weight |
|---|---|---|---|---|
| Logistic | 2 | 92.60 | 13.99 | 0.00 |
| Hill | 2 | 93.33 | 14.71 | 0.00 |
| Richards | 3 | 90.19 | 11.57 | 0.00 |
| Hulin | 4 | 78.61 | 0.00 | 0.99 |
| Double-Richards | 4 | 91.49 | 12.88 | 0.00 |

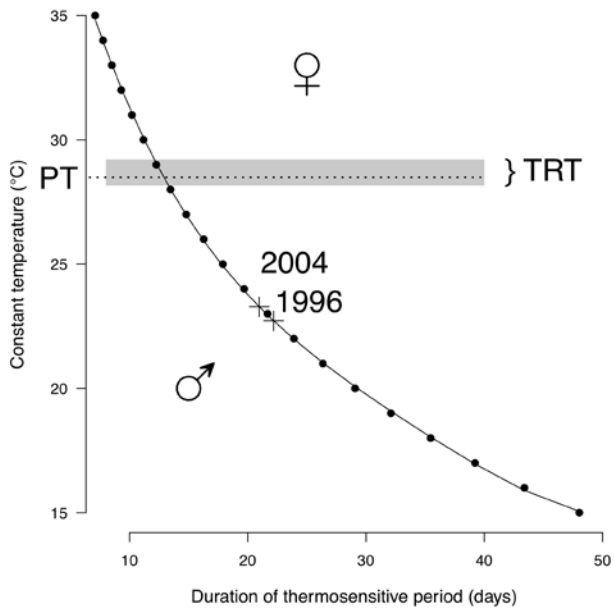


**Fig. 8.** Relationship between constant incubation temperature and duration of the thermosensitive period for sex determination in days based on the modeled thermal reaction norm for embryo growth (see Fig. 6). Temperatures for pivotal temperature (PT) and transitional range of temperatures (TRT) are shown (see Fig. 7)

sex determination. Thus, the effect of a temperature must be weighted by the growth of embryo at this temperature (Pieau 1984, Booth & Freeman 2006). However, this effect is not sufficient to explain the sex ratio bias observed in this experiment (Marc Girondot, unpublished observation).

The incubation duration has been proposed as a proxy for the sex ratio as it integrates the influence of temperature during all the development (Godfrey et al. 1999, Mrosovsky et al. 2009). However, this solution is not entirely satisfactory because the temperature influences incubation duration during all of incubation, whereas temperature influences sex only during the TSP. Thus, one can imagine a situation

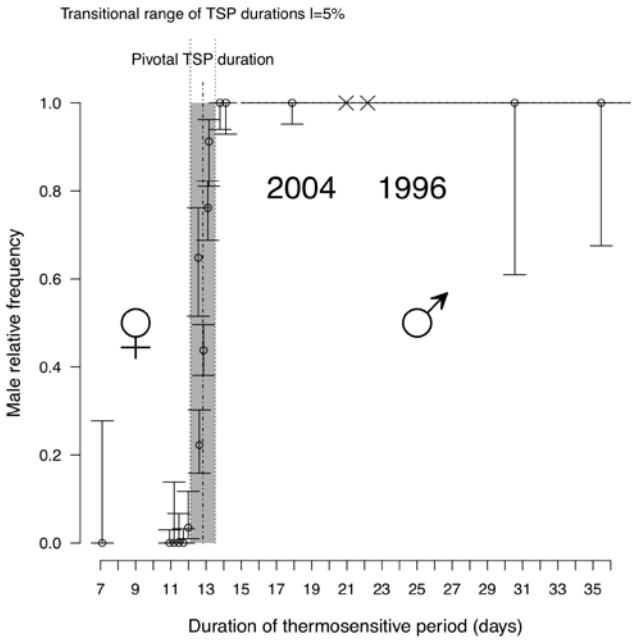


**Fig. 9.** Calibration curve of sex ratio as a function of TSP duration, using incubations at constant temperatures. The error bars represent twice the standard deviation for each point estimate. The line shows the selected model based on Table 2. Arrows indicate the estimated TSP duration for two nests incubated in natural condition from 1996 (Fig. 5A) and 2004 (Fig. 5B) and their corresponding sex ratios

with long (respectively short) incubation duration because the beginning or the end is cold (respectively warm) but the temperature within the TSP is warm (respectively cold). In such a situation, the sex ratio will be estimated wrongly.

An alternative solution is to use the TSP duration as a proxy for sex ratio. TSP duration can be evaluated at constant incubation temperature for calibration using the embryo growth model and thermal reaction norm of embryonic growth rate (see previous section) (Fig. 8). The relationship between TSP duration and sex ratio for these nests incubated at constant temperature has been fitted using the same method as the relationship between temperature and sex ratio (Table 2, Fig. 9). Using this relationship, TSP duration for natural nests can be transformed into sex ratio. The confidence interval for the sex ratio can be obtained based on the confidence interval from the relation used for calibration (Fig. 9). To test the procedure, the length of the TSP for the two nests shown in Fig. 5 was determined and their sex ratio predicted using the calibration curve (100% males, Fig. 9). This prediction is in agreement with the sex ratios actually observed (reviewed by Girondot 1999).

However, the thermal reaction norms for any biological reaction, and of course for embryonic growth rate, are bell shaped with a maximum at an intermediate optimal temperature (Schoolfield et al. 1981). Thus, the same incubation duration is expected below and above this optimal temperature where 100% males or 100% females can be obtained respectively. This situation is unlikely to be observed for *E. orbicularis* thermal reaction norm for embryonic growth because the optimum (temperature at which the embryonic growth rate is maximum) is higher than 35°C (Fig. 6), a temperature that is rarely attained within natural nests (Fig. 2).

We have shown that incubation duration could lead to wrong conclusions about sex ratio due to the non-monotonous trend of temperatures effect on growth rate. The solution that we advocate here using TSP duration is also not fully satisfactory because there is no evidence that the thermal reaction norm for embryo growth is the same as the thermal reaction norm for sexualisation which describe how the temperature affects more or less the sexualisation of the gonad. The best solution would be to convert temperatures within TSP into sex ratio taking into account this new thermal reaction norm for sexualisation. The shape of this reaction norm cannot be deduced from the thermal reaction norm of sex ratio due to the threshold effect of the proportions (a proportion cannot be lower than 0 and higher than 1). For example, 35°C is a more feminizing temperature than 30°C (Pieau 1982), but 100% females are observed at both temperatures. We can predict that the sexualisation thermal reaction norm is non-linear according to temperature as for all thermal reaction norms (Schoolfield et al. 1981).

Incubations at changing temperatures with sexed embryos are necessary to estimate the thermal reaction norm for sexualisation. These data are not still available for *E. orbicularis* and only the TSP duration can be used as a proxy for sex ratio at current time.

# Discussion

The different steps presented in this paper permit one to convert air, soil, nest or TSP duration or temperatures into sex ratio. We used two nests incubated in nature to test the method, but these two nests were 100% masculinized thus the test has low statistical power. It would be better to have more nests and some of them with mixed sex ratio.

The method that we preconize to estimate sex ratio from nest temperatures requires that the thermal reaction norm for embryo growth and the thermal reaction norm for sexualisation are known. The thermal reaction norms are genetically determined

and can be different for different populations based on genetic drift or selection (Angilletta et al. 2003). Selection can be of primary importance because the thermal niche for different populations can be very different depending on the latitude and the climate. As a note of caution, the heterogeneity of nest temperatures is probably lower than the heterogeneity of soil temperatures at the scale of entire ecosystems because turtles do not choose nesting site randomly: they are generally located in sandy soil, in a small hill exposed to the west. Of course, females have plastic behaviour and they can nest in other places when this situation is not available. Thus we encourage the teams working in the field to evaluate these two reaction norms for the populations of their working area rather than using the one described here for *E. orbicularis* from Brenne in the centre of France.

To be in the best situation to estimate sex ratio of natural nests, first it is necessary to get calibration values for the thermal reaction norms for embryo growth and for sexualisation. A set of nests must be monitored using temperature data loggers. The higher the diversity of temperatures both within and between nests, the better the estimates thus a lower number of nests are necessary. For example, even only two nests with large range of temperatures permit one to estimate the thermal reaction norm for embryo growth but the confidence interval was large (Fig. 6). Thus it is better to monitor nests in different soil media, with different exposure and at different times to sample a large range of incubation temperatures. Incubation duration, being defined as the time from egg laying until piping (when the embryo egg tooth breaks the shell) or emergence, must be recorded and juveniles must be sexed for each nest. The higher the number of sexed juveniles, the better. Until recently, the only method for sexing hatchlings was lethal because it requires examining gonads histologically. Laparoscopy could be used only for large individuals but is also lethal for *E. orbicularis* hatchlings (Pieau & Girondot, unpublished). Two recent studies have reported that steroid hormones can be detected in amniotic fluid obtained from eggshells after hatching and differential levels permit to distinguished males and females for eggs incubated at constant incubation temperatures in marine turtles (*Chelonia mydas* and *C. caretta*) (Kobayashi et al. 2015, Xia et al. 2011). This method must be still evaluated in natural conditions. These methods cannot be used at large spatial and temporal scales but could be used to calibrate sexualisation thermal reaction norm and then sex ratio could be determined from time series of nest temperatures.

If the sex ratio cannot be easily determined for a natural nest, the thermal reaction norm for embryo growth rate can be still determined and then TSP duration could be used as a proxy of sex ratio.

All the statistical methods described in this paper are available in two R packages: *HelpersMG* (Girondot 2016b) for meteorological statistical tools and *embryogrowth* (Girondot 2016a) for statistical tools related to thermal reaction norm for embryonic growth and temperature-dependent sex determination. Both packages are available in the Comprehensive R Archive Network (CRAN) website: www.cran.org.

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