

A mechanistic model of temperature-dependent sex determination in a chelonian: the European pond turtle

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Summary

1. In temperature-dependent sex determination (TSD) species, offspring sex-ratio is dependent on the environmental temperature. For oviparous sauropsid species, temperature within the nest influences gonadal sexual differentiation during a small window of embryogenesis called the thermosensitive period (TSP).

2. The absence of sexual dimorphic characteristic in juveniles of TSD species coupled with the lack of a non-invasive method to determine sex is a great obstacle to studies estimating sex-ratios under natural conditions. Some authors have proposed proxies of sex-ratio obtained through correlative approaches. They commonly extrapolate the empirical profile of sex-ratios as a function of constant incubation temperature established for several species in the laboratory to a field context. However, most of these proxies have been refuted by studies realized under field conditions, and consequently they cannot be used to predict sex-ratio under natural conditions.

3. Here, we propose a new thermal model of TSD using a mechanistic approach. We built this model from a collection of published data of physiological processes (i.e. the growth of the embryo, the growth of gonads and the activity of the enzyme aromatase) underlying the TSD mechanism, for the European pond turtle (*Emys orbicularis*). This new approach provides integration of incubation temperature fluctuations, as well as the cumulative and differential effect of high and low temperatures on sexual differentiation to embryo sex determination.

4. The significant consistency obtained between observed and predicted sex-ratios both at diverse constant and fluctuating incubation temperatures provides hope to develop an efficient method to predict sex-ratio under natural conditions. The reliable validity of this new model could have wide-ranging implications for the understanding of the TSD mechanism, as well as its evolutionary and ecological consequences in natural populations.

Key-words: fluctuating temperature, mechanistic model, sex-ratio, TSD, turtle

Introduction

Two different sex determination systems co-exist in sauropsids (Bull 1980; Ewert, Jackson & Nelson 1994). In genotypic sex determination (GSD) system, information contained in sex chromosomes determines embryo sex. In contrast, in temperature-dependent sex determination (TSD) system, a form of environmental sex determination (ESD), incubation temperature appears to be the most critical factor affecting gonadal differentiation (Bull 1980; Raynaud & Pieau 1985). For oviparous TSD species, sexual differentiation is dependent on the environmental temperature within the nest during a precise

window of embryogenesis called the thermosensitive period or TSP (Pieau & Dorizzi 1981; Mrosovsky & Pieau 1991). The sex of the embryo is irreversibly determined at the end of the TSP (Raynaud & Pieau 1985). To date, TSD is unknown in snakes and birds, but occurs in all studied crocodylians and tuataras, and is prevalent in turtles and less frequent in lizards.

The TSD mechanism was formerly described by defining three different patterns according to the sex-ratio produced as a function of constant incubation temperatures (Ewert *et al.* 1994). In the MF (Male–Female) or TSD Ia pattern, low temperatures produce males and high temperatures produce females. The opposite is true for the FM (Female–Male) or TSD Ib pattern. In the FMF (Female–Male–Female) or TSD II pattern, low and high temperatures produce females, while

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intermediate temperatures produce males. In all patterns, both sexes are produced in equal proportions at a pivotal temperature (P) and in variable proportions in the transitional range of temperatures or TRT (Mrosovsky & Pieau 1991). Besides their fundamental interest, these theoretical TSD patterns are not representative of the TSD mechanism under fluctuating temperature regimes as encountered in natural nests (Georges, Limpus & Stoutjesdijk 1994).

The lack of knowledge of the exact involvement of temperature on sex determination, as well as the absence of any non-invasive method to estimate sex-ratios in natural conditions, is one of the great obstacles to understand the evolutionary and ecological significance of TSD.

In TSD species, and particularly in turtles, no easy method of determining offspring sex is currently available. Juveniles do not possess sexually dimorphic characteristics. The only method to precisely determine sexual phenotype is invasive; by the direct observation of gonadal morphology (Yntema 1976). Today, one of the major challenges in studies of TSD species is to find a simple way of sexing juveniles emerging from natural nests and estimating sex-ratios. Some authors have proposed proxies of sexual determination and tried to extrapolate results from laboratory experiments to a field context. The mean incubation temperature (Bull 1980), the combination of the mean and thermal variance (Bull 1985; Souza & Vogt 1994), the duration spent above and below P (Pieau 1982; Valenzuela, Botero & Martinez 1997) or the incubation duration (Marcovaldi, Godfrey & Mrosovsky 1997) were consecutively proposed to predict natural sex-ratios. However, most of these proxies, defined by correlative approaches, were refuted by studies realized under field conditions (Valenzuela 2001; Georges *et al.* 2004). Other authors suggested that it is due to a crucial role of thermal fluctuations in the TSD mechanism with a differential effect of high and low temperatures on embryonic development and gonadal differentiation. Facing limitations of pure correlative approaches, there have been attempts at devising and testing new models that incorporated mechanistic insights of TSD (Georges 1989; Georges *et al.* 1994; Valenzuela 2001). The most reliable model, developed by Georges *et al.* (1994), supposes that females will be produced if more than half of embryonic development occurs above the pivotal temperature during the TSP, otherwise males will be produced. Under this hypothesis, the model translates fluctuating temperatures into constant temperature equivalents (CTE) and predicts the sex-ratio as equivalent to that in a constant temperature incubator set at the value of the CTE. However, the CTE approach is limited to cases of temperature fluctuations with a constant magnitude about a stationary mean, while temperature variations are much more complex in natural nests (Georges *et al.* 2004). This is due to the lack of sufficient studies concerning sex-ratio performed under fluctuating temperatures and consequently to the poorly understood relationships between temperature and sexual outcomes in TSD species (Valenzuela 2001). It is now crucial to understand the real influence of incubation temperature and its fluctuations on the different processes involved in sex determination mechanism. This latter step

might provide a new way to accurately predict sex-ratios under natural conditions (Wibbels, Bull & Crews 1994; Pieau, Dorizzi & Richard-Mercier 1999).

Here, we propose a new model of TSD using a complete mechanistic approach. Molecular and physiological bases of TSD have been well established in several sauropsids species (Deeming & Ferguson 1989; Wibbels *et al.* 1994; Pieau & Dorizzi 2004). In this new process-oriented model, we considered physiological processes underlying TSD and simulated their interplay with temperature fluctuations during incubation to predict embryo sex. The model was built using several physiological variables (i.e. the growth of the embryo, the growth of gonads and the activity of the enzyme aromatase) collected for the European pond turtle, *Emys orbicularis* (Linné, 1758), the most studied species regarding to TSD mechanism (Pieau 1982; Pieau *et al.* 1999). We tested the validity of model predictions according to various sex-ratio data: (i) the well-known sex-ratio profile of *E. orbicularis* at constant incubation temperatures; and (ii) the observed sex-ratios yielded at several fluctuating temperature regimes. We provided evidence that such mechanistic approach is needed and compared sex-ratio estimates provided by our model to those estimated with the widely accepted CTE model. Finally, we discussed the limitations of the model, as well as its generalization to other TSD species. We also postulate at the wide-ranging implications of this new model for the understanding of the TSD mechanism, and its evolutionary and ecological consequences in natural populations.

Materials and methods

TSD MECHANISM KNOWLEDGE

The TSD mechanism is characterized by the existence of a TSP that corresponds to the first stages of gonadal differentiation, these embryonic stages being approximately the same whatever TSD species mass (Bull 1987). It is possible to delimit TSP from species-specific weights of the embryo at the stages corresponding to the beginning and the end of this period.

A variety of experiments performed in diverse TSD species provided evidence for oestrogen's involvement in gonadal sex differentiation. The feminizing role of endogenous oestrogen in the gonad has been shown during the TSP (Pieau & Dorizzi 2004). Some studies revealed that levels of gonadal oestrogens are correlated with the gonadal activity of aromatase (i.e. the enzyme complex that converts androgens to oestrogens) during the TSP (Dorizzi *et al.* 1994; Jeyasuria & Place 1997). Moreover, gonadal differentiation is in agreement with the aromatase activity pattern (Pieau *et al.* 1998).

Incubation temperature acts on embryonic growth throughout the entire incubation period with a differential effect of high and low temperatures on developmental rate (Pieau & Dorizzi 1981; Deeming & Ferguson 1989). Temperature also has a cumulative effect on aromatase activity, as well as on the pattern of gonadal differentiation during the TSP (Desvages & Pieau 1992; Georges *et al.* 1994). Finally, to define a reliable predictor of embryo sex, we used results obtained for another related sauropsid species with GSD, the chicken, to consider aromatase activity per unit of gonad as a good index of sex at the end of the TSP (Vaillant *et al.* 2001).

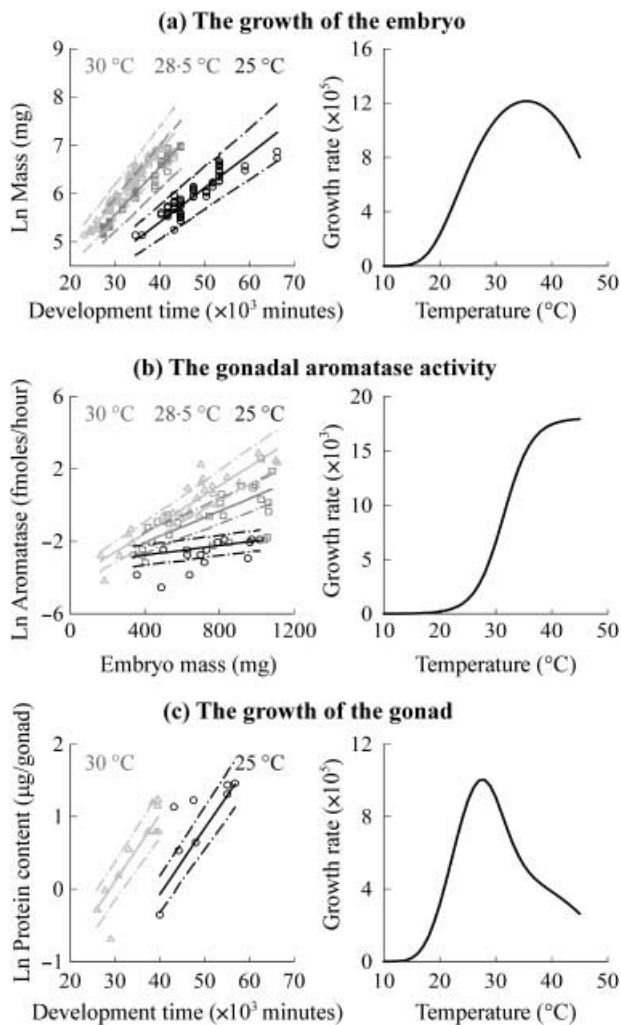


Fig. 1. Mathematical models fitted on empirical physiological data obtained from incubations of *E. orbicularis* eggs at different constant temperatures (25 °C, 28.5 °C and 30 °C): (a) the growth of the embryo; (b) the gonadal activity of the enzyme aromatase; and (c) the growth of the gonad. On the left: fitted functions (solid lines) and their confidence interval (dashed lines); on the right: relative simulated intrinsic growth rate dependent on incubation temperature.

PHYSIOLOGICAL DATA

The European pond turtle, *E. orbicularis*, exhibits a TSD Ia pattern with 100% of males yielded at 25 °C, 100% of females yielded at 30 °C (Girondot 1999) and a pivotal temperature estimated at 28.62 °C (Godfrey, Delmas & Girondot 2003). TSP has been shown to overlap stages 16–22 of embryogenesis (Yntema 1968) corresponding to an embryo mass between 170 and 1100 mg (Pieau & Dorizzi 1981). Three different types of physiological and molecular data, collected from eggs of *E. orbicularis* incubated at various constant temperatures, were utilized to develop our mechanistic model:

1. The mass of embryo (mg) according to development time, obtained from eggs incubated respectively at 25 °C, 28.5 °C and 30 °C (Fig. 1a; Pieau & Dorizzi 1981).
2. The gonadal aromatase activity (fmoles per hour per gonad) according to embryo mass, obtained from eggs incubated, respectively, at 25 °C, 28.5 °C and 30 °C (Fig. 1b; Pieau *et al.* 1998).

3. The gonadal protein content (µmoles per gonad) according to embryo mass, obtained from eggs incubated, respectively, at 25 °C and 30 °C (Fig. 1c; Pieau *et al.* 1998).

MATHEMATICAL STRUCTURE OF THE MODEL

For each set of raw data, we adopted the model selection strategy recommended by Burnham & Anderson (1998) for selecting the best approximating model from a set of candidates based on the minimum Akaike's Information Criteria or AIC (Akaike 1974). If L was the maximum-likelihood for a specific model using k independently estimated parameters, then $AIC = -2 \ln L + 2k$.

The growth of the embryo

In poikilothermic species, early mass of the embryo is commonly modelled as varying exponentially with time of development (West, Brown & Enquist 2004). Among functions of the exponential family, we selected the following equation (1) to model the growth of the embryo:

$$m(t) = m_0 \exp(r_m t) \quad \text{eqn 1}$$

where m_0 is the initial embryo mass at $t = 0$, r_m is the embryo growth rate and t is the development time. Based on experiments in several sauropsid species, we assumed that embryo growth rate r_m could be represented as a logistic function dependent on incubation temperature (Pieau & Dorizzi 1981; Bardsley *et al.* 1995). Moreover, r_m should approach 0 at very low temperatures and follow a bell-shaped curve to model the harmful impact of too high temperatures (Georges *et al.* 2005). The most widely accepted model of poikilotherm development was proposed by Sharpe & DeMichelle (1977): it describes the nonlinear response of developmental rate depending on incubation temperature. Nevertheless, this deterministic model is very demanding of data and requires estimates of developmental rates for temperatures outside the range of the constant temperatures that support successful developments (Georges *et al.* 2005). Such data are not yet available because of the impossibility to be obtained by constant-temperature experiments alone. So, we used a similar function that provides much more control on parameters that influence the expected shape of the relation. The growth rate r_m was modelled as the product of two scaled Richards' functions with eqn (2):

$$A = \exp(U_m)$$

$$B = 2^A - 1$$

$$C = 1 + B^2 \exp\left(\frac{-\Delta X_m}{Y_m}\right)$$

$$r_m = \frac{M_{\text{Max}}}{[C + B(\exp((1/Y_m)(X_m - T)) + \exp((1/Y_m)(T - X_m - \Delta X_m)))]^A} \quad \text{eqn 2}$$

This function included five parameters: X_m , ΔX_m , U_m , Y_m and M_{Max} . It modelled r_m rising from 0 to the scaling parameter M_{Max} and further declining from M_{Max} to 0 as incubation temperature T increases. We could impose an increasing growth rate between 20 °C and 35 °C, and a decreasing growth rate above 35 °C (C. Pieau, pers. obs.): the

first inflexion point (between 0 and M_{Max}) is observed at incubation temperature $T = X_m$ and the second (between M_{Max} and 0) at incubation temperature $T = X_m + \Delta X_m$. The change rate of r_m at inflexion points is dependent on the Y_m parameter and the U_m value, which determines the asymmetry of the function (i.e. symmetric around X_m and $X_m + \Delta X_m$ for $U_m = 0$, or asymmetric for $U_m \neq 0$).

Aromatase activity during the TSP

In *E. orbicularis*, it has been shown that aromatase activity a increases according to embryo mass during the TSP (Pieau *et al.* 1998). Among possible functions we selected the following exponential function (3) to model the gonadal aromatase activity:

$$a(m) = a_0 \exp(r_a(m - m_{isp})) \quad \text{eqn 3}$$

where a_0 is the initial gonadal aromatase activity, r_a is the growth rate of aromatase activity and m is the mass of the embryo. As aromatase activity increases from the beginning of the TSP, an origin shift was performed, represented here by $(m - m_{isp})$, where m_{isp} is embryo mass at this starting time (i.e. embryo mass of 170 mg). Experimental data showed that aromatase activity remains very low in the gonad at 25 °C, while it increases at 28.5 °C and 30 °C during the TSP, with a higher activity at the latter incubation temperature (Pieau *et al.* 1998). These data are concordant with the increasing feminization (through oestrogen levels) of the gonads with increasing temperature within the bounds of constant temperatures that support development. We finally decided to model the growth rate r_a as increasing with temperature and reaching thresholds at very low and very high temperatures. The effect of incubation temperature upon aromatase activity growth rate r_a was modelled as a scaled sigmoid logistic function (4):

$$r_a = \frac{A_{Max}}{1 + \exp\left(\frac{X_a - T}{Y_a}\right)} \quad \text{eqn 4}$$

This function used three parameters: X_a , Y_a and the scaling parameter A_{Max} . It was adjusted to allow growth rate r_a to be approximately 0 at very low temperatures (< 20 °C) and to increase until a threshold A_{Max} reached at higher temperatures (> 40 °C): these conditions are coherent with measures of gonadal aromatase performed on the sea bass (Gonzalez & Piferrer 2002). The inflexion point of r_a is reached at $T = X_a$ and the change rate at this point is dependent on the parameter Y_a .

The growth of the gonad during the TSP

The growth of the gonad, estimated by protein content, seems also to exponentially increase with embryo mass and development time during the TSP (Pieau *et al.* 1998). We selected the following exponential function (5) to describe gonadal growth

$$g(t) = g_0 \exp(r_g(t - t_{isp})) \quad \text{eqn 5}$$

where g_0 is the initial protein content in the gonad, r_g is the gonadal growth rate and t is the time of development. Here, t corresponds to embryo mass $m(t)$ in raw data and was re-calculated from eqn (1). As gonads appear at the beginning of the TSP (i.e. t_{isp}), we also performed an origin shift, represented here by $(t - t_{isp})$, to consider only the time from this starting time.

Based on our biological knowledge, we assumed that gonadal growth rate r_g could be represented as the combination of the growth of the embryo and the influence of oestrogen levels on gonadal differentiation through aromatase activity. At 25 °C, aromatase activity is low and permits a continuous growth of the gonad towards the testicular way, while at 30 °C increasing aromatase activity inhibits testicular development before permitting ovarian structures differentiation (Pieau *et al.* 1999). So, the growth rate r_g was modelled as a classical logistic function (6):

$$r_g = \frac{r_m}{1 + \exp\left(\frac{X_g - r_{Eq,a}}{Y_g}\right)} \quad \text{eqn 6}$$

This function used two parameters, X_g and Y_g and included the growth rate of the sharing embryo r_m scaled by the potential inhibition of gonadal growth by oestrogen levels, represented by the equivalent aromatase growth rate $r_{Eq,a}$. $r_{Eq,a}$ is calculated from eqn (4) using the embryo mass m corresponding in raw data and the aromatase activity a at the previous time step. The two parameters X_g and Y_g control, respectively, the inflexion point and the shape of the function r_g .

FITTING PARAMETERS PROCEDURE

The values of parameters were estimated from raw physiological data and not from observed sex-ratios. For each function, parameters value, that best fitted the natural logarithmic transformed observed data, was searched for using maximum-likelihood estimation with a Gaussian error. The mean value m and the standard deviation SD of each parameter were fitted. Using ± 2 SD as the range of values for each parameter, it was possible to estimate the range of observed variation for each kind of physiological data. This was used to estimate the likelihood of each observation. Such a procedure has the advantage of accounting for potential heteroskedasticity. Generalized reduced gradient (GRG2) was used as a fitting algorithm (Lasdon *et al.* 1978).

The theoretical sexual threshold, represented by aromatase activity per unit of gonad (i.e. protein content) at the end of the TSP, was obtained from eqns (3) and (5) for a temperature of 28.62 °C (i.e. pivotal temperature that theoretically yields 50% of each sex) and an embryo mass of 1100 mg (i.e. the end of the TSP).

MODEL SIMULATION PROCEDURE

The functions (1), (3) and (5) and their relative growth rates dependent on incubation temperature are continuous because they are calculated from data obtained at constant temperatures. However, our aim was to simulate sex determination of embryos virtually incubated under fluctuating temperature regimes. For each equation, we defined a new discrete growth rate that allowed integration of temperature fluctuations in sex differentiation mechanism. Finally, we calculated, step by step, consecutive values $y_{t+\Delta t}$ for each physiological process ($m(t)$, $a(m)$ and $g(t)$) during incubation with eqn (7), for each time step Δt and without loss of precision:

$$y_{t+\Delta t} = y_t + y_t(\exp(r\Delta x) - 1) \quad \text{eqn 7}$$

where r is the growth rate of each function (r_m , r_a or r_g) and x corresponds to development time or embryo mass depending on concerned data and function.

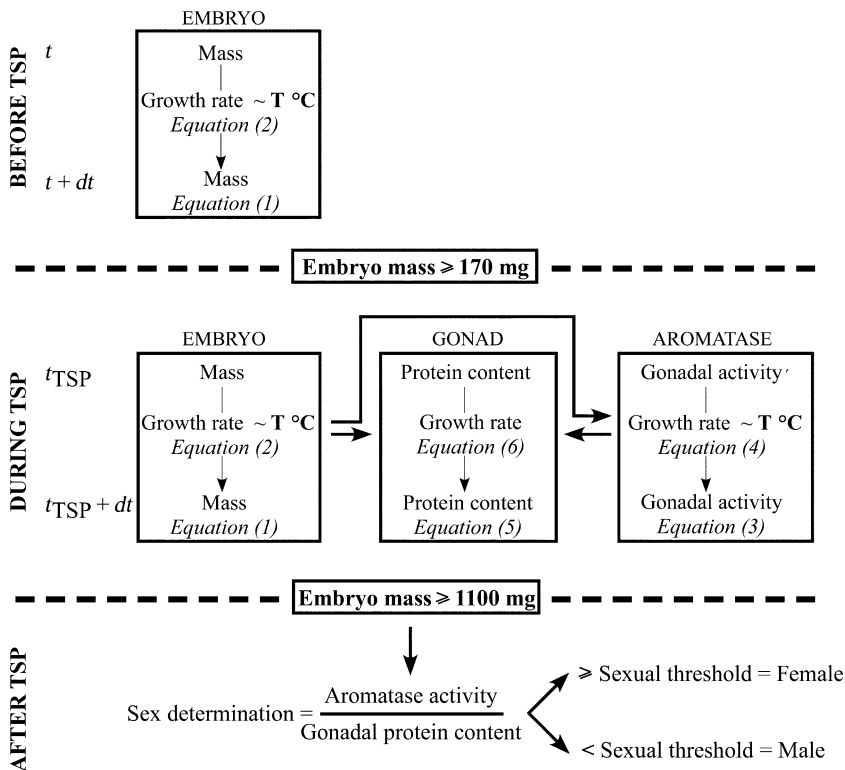


Fig. 2. Simulation procedure of the physiological processes modelled in the mechanistic model of TSD, at each time step ($t + dt$) before, during and after the thermosensitive period (TSP). Related equations described in the manuscript, the influence of incubation temperature and interactions between physiological processes are specified.

For each simulation (i.e. each embryo), the values of parameters used in functions (1)–(6) are determined among the range of fitted values from a normal distribution $N(m, SD)$, representative of a variation in individual characteristics. Simulations of embryos growth permit us to precisely delimit the TSP in which aromatase activity and the growth of the gonad are subsequently modelled. The calculated value of aromatase activity per unit of gonad at the end of TSP determines embryos sex. Females are produced for a value superior to our threshold and males are produced in any other case (Fig. 2).

We developed the mechanistic model in R programming language (Appendix S1) version 2.5.0 (R Development Core Team 2007).

SOURCE DATA AND VALIDITY TEST OF THE MECHANISTIC MODEL

To test the validity of the model, we compared results of sex-ratios obtained from incubations of *E. orbicularis* eggs with model predictions. Two kinds of data were utilized to perform this test. We used the known TSD Ia sex-ratio profile of *E. orbicularis* (Girondot 1999). This profile was established from incubations of 1288 eggs at 16 constant temperatures realized under laboratory conditions over the last 25 years (Fig. 3a). No empirical data of sex-ratios was available from incubation of eggs at fluctuating temperatures and so we incubated eggs of *E. orbicularis* at different fluctuating temperature regimes during three consecutive years (2002–2004) under laboratory conditions (Table 1). Six of these regimes were daily sinusoidal with a constant range about a stationary mean temperature, and four (A–D) were inspired by records in natural nests of *E. orbicularis* (Pieau 1982), with heterogeneous variations of temperature (Supplementary Fig. S1). Gravid females of *E. orbicularis* were captured in the 'Parc Naturel Régional de la Brenne'. Eggs were collected from

females where oviposition was induced by an intramuscular injection of 2 IU of oxytocin (Ewert & Legler 1978). Incubations were realized in programmable incubators (Memmert™, IPP 200–400, Schwabach, Germany). Eggs were placed in plastic boxes and three-fourth buried in vermiculite with a controlled and intermediate water potential (-398 kPa: 0.44 g of sterilized water per gram of vermiculite) maintained constant by water addition during all the incubation period. Eggs from the same clutch (i.e. 8–12 eggs) were dispersed as much as possible between the temperature regimes to prevent clutch effects. After hatching, juvenile sex was determined by dissection and microscopic observation of gonadal morphology.

EVALUATION OF THE CTE MODEL

We tested the predicted sex-ratios from the CTE model on our sex-ratio data for constant and fluctuating thermal regimes with a constant magnitude about a stationary mean (Table 1). Following the method developed by Georges *et al.* (1994), we estimated the minimum development temperature (T_0) for CTE calculation by regressing developmental rate and constant temperature from our physiological data (y intercept = T_0). We calculated the CTE within the TSP, delimited by specific mass of embryo, and estimated sex-ratio from the established linear relationship between sex-ratio and constant incubation temperature (Table 2). We could compare sex-ratio estimates derived from the CTE model with sex-ratio predicted with our mechanistic approach in estimating the likelihood of the observations for each model and then their AIC weight (w_i). w_i is considered as the weight of evidence in favour of the model i (Burnham & Anderson 1998).

Finally, we had opportunity to test the underlying hypothesis of the CTE model, which predicts that the proportion of embryonic development above P is a good proxy of embryo sex. We ran 100

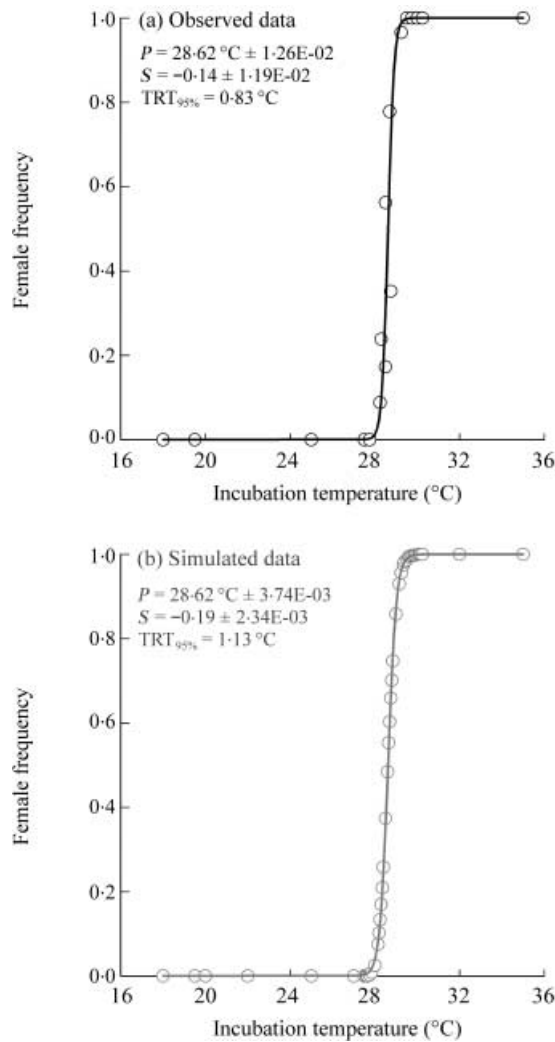


Fig. 3. Sex-ratio profiles of *E. orbicularis* expressed as female frequency dependent on constant incubation temperature. (a) the profile fitted on observed sex-ratios obtained from incubation of 1288 eggs at 16 constant temperature (Girondot 1999); and (b) the profile fitted on predicted sex-ratios obtained from model simulations. For each profile, mean values \pm SE of the pivotal temperature P , the slope of the function S and the transitional range of temperatures, $TRT_{95\%}$ are specified.

simulations (i.e. 100 embryos) of our mechanistic model and estimated this proportion (from eqn (1) of our mechanistic model) by dividing the mean sum of gains in embryo mass that occurred above P per the total gain in embryo mass during the TSP.

Results

FITTING EQUATIONS AND PARAMETERS

For the three kinds of physiological processes, an exponential function was defined as the best model to represent the raw data (Supplementary Table S1). We observed that all the fitting equations and parameters appropriately described the three kinds of physiological mechanism patterns including an intrinsic growth rate as a function of incubation temperature (Fig. 1). Moreover, the range of fitted parameters values allows us to also describe observed variance for each physiological process (Fig. 1).

THE MECHANISTIC MODEL

Predictions of the TSD model was statistically tested using a paired t test between observed and predicted proportions of females that were arcsine-transformed. There was no significant difference between the observed sex-ratios and those predicted from the model at a 5% level ($df = 24$, $t = -0.16$, $P = 0.878$) both at constant and fluctuating incubation temperatures with constant and non-constant thermal variances (Fig. 4).

We could reproduce the TSD Ia sex-ratio profile of *E. orbicularis* from simulations of 5000 eggs per constant temperature within a range of 20 °C up to 35 °C with a step of 0.5 °C. Using the method developed by Godfrey *et al.* (2003), we adjusted the best mathematical function to describe the sex-ratio profile and estimated its parameters values (P , S and TRT). A profile with a pivotal temperature $P = 28.62 \pm 3.74 \cdot 10^{-3}$ °C, a slope $S = -0.19 \pm 2.34 \cdot 10^{-3}$ and a range of temperatures that produce 5%–95% of females $TRT_{95\%} = 1.13$ °C (Fig. 3b) was obtained, consistent with the known observed sex-ratio profile (Fig. 3a).

We also demonstrated the feminizing role of thermal variance proposed by Georges *et al.* (1994). We simulated incubation of 5000 eggs under sinusoidal temperature regimes with mean temperature ranging from 25 °C to 30 °C and a related thermal variance ranging from 0 °C to 5 °C. We verified that differential effects of high and low temperatures on developmental rates yield a proportion of females that increases with the thermal variance for eggs incubated at the same mean temperature (Fig. 5).

Table 1. Observed sex-ratios (SR_f), expressed as the frequency of females, obtained from incubations of *E. orbicularis* eggs performed at the laboratory under various fluctuating temperature regimes. In 2002 and 2003, temperature regimes were 24 h-sinusoidal with a constant range of temperature, while in 2004, temperature regimes were fluctuating with heterogeneous variations in temperature (Supplementary Fig. S1)

Regime	2002			2003			2004			
	26 ± 3.5 °C	28 ± 0.9 °C	27 ± 2.6 °C	29 ± 3 °C	28.5 ± 5 °C	28.5 ± 1.5 °C	A	B	C	D
Males	17	14	18	3	2	21	1	0	0	0
Females	0	0	0	30	29	11	21	23	22	21
SR_f	0	0	0	0.91	0.93	0.34	0.95	1	1	1

Table 2. Summary of models' simulations and predictions of sex-ratio (SR_f), expressed as the frequency of females, for two kinds of fluctuating regimes of incubation (with constant vs. heterogeneous daily variations). With our mechanistic model, we estimated the proportion of embryonic development (Dev. Prop.) that occurred above P during the TSP and the associated sex-ratio. With the CTE model, we could only estimated the CTE value and the associated sex-ratio in the case of regimes with constant variations about a stationary mean

Regime	Thermal variance	Mechanistic model		CTE model	
		Dev. Prop.	Predicted SR_f	CTE	Predicted SR_f
26 ± 3.5 °C	Constant	0.467	0	27.52	0
28 ± 0.9 °C	Constant	0.364	0.05	28.09	0.15
27 ± 2.6 °C	Constant	0.419	0	27.79	0
29 ± 3 °C	Constant	0.685	0.99	29.90	1
28.5 ± 5 °C	Constant	0.553	0.99	30.83	1
28.5 ± 1.5 °C	Constant	0.429	0.65	28.76	0.61
A	Heterogeneous	0.461	0.93	–	–
B	Heterogeneous	0.483	0.95	–	–
C	Heterogeneous	0.542	0.99	–	–
D	Heterogeneous	0.488	0.97	–	–

Note: For the CTE model, we used the method developed by Georges *et al.* (1994). We estimated the developmental zero for *E. orbicularis* at $T_0 = 18.96$ °C ($R^2 = 0.96$, $n = 3$) and estimated sex-ratio following the established relation between frequency of females (SR_f) and constant temperature (T) as:

$$SR_f = 0 \text{ for } T < 27.86 \text{ °C}$$

$$SR_f = 0.678 T - 18.896 \text{ for } 27.86 < T < 29.33 \text{ °C } (R^2 = 0.89, n = 15)$$

$$SR_f = 1 \text{ for } T > 29.33 \text{ °C.}$$

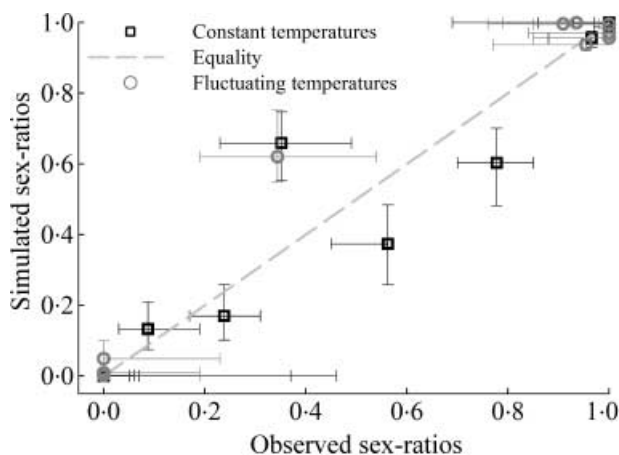


Fig. 4. Observed vs. simulated sex-ratios, expressed as the frequency of females. The dashed line represents the perfect equality between the two kinds of data. For each point, standard deviations are figured as representing: (i) horizontally, errors related to temperature records; and (ii) vertically, errors related to number of eggs incubated.

THE CTE MODEL

Considering the CTE model, we found no significant difference at a 5% level between observed sex-ratios and those predicted from the model (Table 2), applied on constant and daily sinusoidal cycles of temperature (paired t test: $df = 20$, $t = 1.71$, $P = 0.102$). Nevertheless, AIC weights provided evidence that the best-approximating model on our sex-ratio data was our mechanistic model ($w_i = 0.99$). Moreover, we showed that the proportion of embryonic development above P during the TSP was not a good predictor of offspring sex (logistic regression: $df = 1$, $t = 1.119$, $P = 0.296$) when tested on all fluctuating temperature regimes (Table 2).

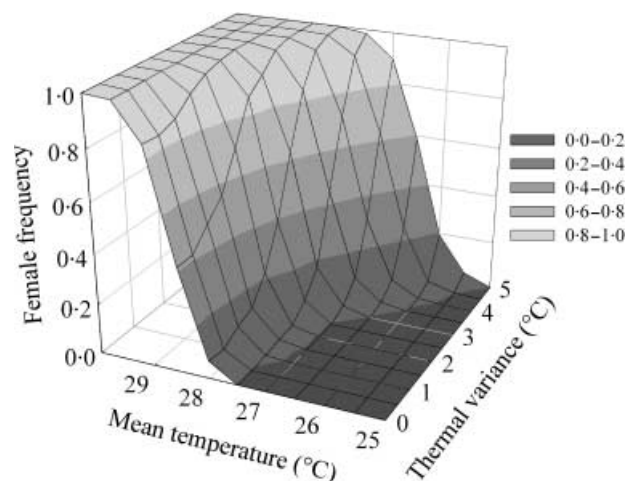


Fig. 5. Predictions of female frequency as a function of mean incubation temperature and associated thermal variance. These results were obtained from simulation of 5000 eggs per 24-h sinusoidal temperature regime.

Discussion

Here, we provide a new method that successfully predicts the sex of embryos (i.e. sex-ratio of a clutch) whatever the thermal regime of incubation (Figs 3 and 4), for a TSD Ia species, the European pond turtle (*E. orbicularis*). To date, only correlative and few other statistical approaches have been proposed in several TSD species to predict sex. Such thermal models of TSD have several limitations and cannot be used to accurately predict sex-ratios under natural conditions (Georges *et al.* 2004). Although AIC weight showed that our model was the most strongly supported, we acknowledge that the CTE model proposed by Georges *et al.* (1994) predicts sex-ratio

efficiently. However, it is limited to cases of fluctuations of constant magnitude about a stationary mean temperature (Table 2). Moreover, simulations of the mechanistic model demonstrated that the hypothesis underlying the CTE model was violated under thermal regimes with heterogeneous variations. Indeed, we showed it is possible to obtain only females even if the proportion of embryonic development above P is inferior to 0.5 (regimes A, B & D in Table 2). These results confirmed that the approach developed by Georges may adequately integrate the effects of mean temperature and periodic daily fluctuations on sexual differentiation. Unfortunately, it does not integrate the effects of aperiodic fluctuations with a non-constant range of temperature during the TSP, the more realistic conditions encountered in natural nests and particularly for shallow-nesting species.

Sex determination is certainly not the real target of incubation temperature but the result of a physiological and molecular processes cascade that is temperature-dependent (Pieau & Dorizzi 2004). Therefore, we adopted a mechanistic approach of TSD and used physiological data collected at constant incubation temperatures to develop a new thermal model of TSD. For the first time, we succeed in combining the effects of incubation temperature and its fluctuations on three interacting physiological processes (the growth of the embryo, the growth of gonads and oestrogen hormone pattern), all involved in the TSD mechanism (Fig. 1; Pieau & Dorizzi 2004), to predict embryo sex. This new approach involved several key challenges: (i) to formalize the relationships between fluctuating temperatures and sex determination from data obtained at constant incubation temperatures; (ii) to consider the cumulative effect of temperatures on sexual differentiation (Georges *et al.* 2004); and (iii) to model the influence and differential effect of high and low temperatures on developmental rates (Georges *et al.* 1994). This latter step is crucial to be able to precisely delimit the period (TSP) in which only temperature exerts its influence on sex determination. To date, no non-invasive method permits accurate estimation of the TSP. At constant incubation temperatures, all rates of physiological processes can be approximated by a linear function of temperature, comprised within a range of acceptable temperatures that permit a good embryonic development. Within these particular conditions, the TSP matches the middle-third of incubation period in both time and stage of development (Pieau & Dorizzi 1981; Yntema & Mrosovsky 1982). Most of authors have used this definition of TSP to propose proxies of sex in TSD species. However, under the complex thermal regimes of natural nests, the cumulative and differential effects of temperatures on developmental rates, uncouple incubation duration and the progression of embryonic stages. If TSP corresponds to the same embryonic stages, its temporal duration and location will shift dramatically depending on the thermal regime experienced.

In our new thermal model of TSD, we defined a discrete logistic growth rate for each fitted exponential function to enable summation, step by step, of the differential and cumulative effects of temperatures on developmental rates. The modelling of embryonic growth dependent on thermal

regime allows us to precisely determine the timing of the TSP corresponding to a species-specific range of embryo masses. During the TSP, the gonad grows at the same time as the embryo and its sexual differentiation is controlled by oestrogen levels through endogenous aromatase activity, which is temperature-dependent (Fig. 2). The differentiation of growth trajectories can be attributed to a complex interaction between deterministic and stochastic factors. The deterministic factors predispose an organism toward a specific growth shape, whereas the stochastic factors modify it in response to the environment the organism experiences. For each simulated embryo, a set of parameters for each function was defined within a range of possible values based on empirical data. This simulated inter-individual variability enabled us to take into account stochastic factors like maternal effects (Bowden, Ewert & Nelson 2000) represented, for example, by initial oestrogen levels allocated by females to their eggs [i.e. parameter a_0 in eqn (3)] or by initial embryo mass [parameter m_0 in eqn (1)], as well as a potential genetic factor in TSD mechanism (Pieau *et al.* 1999). Finally, the model enables production of both sexes in the same thermal regimes.

Without ever using sex-ratio data to build the TSD model, we succeed in reproducing a sex-ratio profile of *E. orbicularis* at constant incubation temperatures (Fig. 3) and predicting sex-ratios obtained under sinusoidal and realistic fluctuating temperature regimes (Table 1, Fig. 4). Moreover, results of model simulations were consistent with the increasing of offspring feminization with thermal variance (Fig. 5), already observed by Georges *et al.* (1994) in another TSD turtle species, *Caretta caretta*. All these significant consistencies with empirical data validate our present physiological and molecular knowledge of TSD, and provide hope to reconcile laboratory with field data. A complete mechanistic approach of TSD has never been realized before, and this kind of model offers new perspectives compared to previous correlative and statistical approaches. For the first time, a model succeeds in integrating daily aperiodic variations with a non-constant range of temperature, as well as the combined effects of deterministic and stochastic factors to predict sex. In addition to the perspective of a real predictive value, this model may become a key tool in natural population studies with a wide range of ecological and evolutionary implications.

Nevertheless, our model has yet some limitations and further work is imperative to validate model predictions with sex-ratio data obtained under real natural conditions. Indeed, we used combined data collected in a temperature range of 20 °C to 35 °C, those bounds corresponding to constant temperatures that support embryogenesis for *E. orbicularis* (Pieau 1982). We know that temperature may fluctuate, for brief exposure, out of these bounds in natural nests. Our extrapolation of relations between developmental rates and temperatures outside of this range (Fig. 1) was constrained by available empirical data and knowledge that could lead to bias in sex-ratio predictions under natural conditions. Particularly, the lack of data for aromatase activity out of these bounds constrained us to model its relation to temperature as a sigmoid curve. A more realistic model might be one similar

to the model of embryo growth rate with an inhibition effect of too high temperatures. Therefore, we acknowledge that further studies will be necessary to precise the relation between aromatase activity and temperature out of these bounds. Data of natural sex-ratios would also provide the chance to re-adjust model mechanism through correction of certain parameters values.

Conclusions

Our new model of TSD has several advantages. On one hand, it provides a non-invasive method and hope to accurately predict offspring sex-ratios under natural conditions. Its predictive role is particularly crucial for studies of species with small clutch size or under conservation concern. On the other hand, this model provides a precise descriptive approach that would be useful to improve our knowledge of the TSD mechanism, as well as the influence of temperature during incubation on a diversity of other traits, especially post-hatching traits. It may be possible to observe physiological responses at a precise period in embryogenesis delimited in time by simulation of the growth of the embryo and correlate these responses to diverse traits. Our mechanistic model may also allow us to explore how TSD species might respond to climatic change, especially in identifying parameters that might be under selection or manipulated by species to ensure development of both sexes in natural nests. Finally, it is planned to generalize our mechanistic model to other TSD species of turtles with TSD Ia and even TSD II patterns. The underlying hypothesis is that TSD mechanism is approximately similar in all TSD species. We suppose that our results validated the structure of the mechanistic model. We could therefore adapt this model to other species in modifying some parameters from new fit on empirical data of the TSD profiles.

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References

- Akaike, H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716–723.
- Bardsley, W.G., Ackerman, R.A., Bukhari, N.A.S., Deeming, D.C. & Ferguson, M.W.J. (1995) Mathematical models for growth in alligator (*Alligator mississippiensis*) embryos developing at different incubation temperatures. *Journal of Anatomy*, **187**, 181–190.
- Bowden, R.M., Ewert, M.A. & Nelson, C.E. (2000) Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proceedings of the Royal Society of London*, **267**, 1745–1749.
- Bull, J.J. (1980) Sex determination in reptiles. *Quarterly Review of Biology*, **55**, 3–21.
- Bull, J.J. (1985) Sex ratio and nest temperature in turtles: comparing field and laboratory data. *Ecology*, **66**, 1115–1122.
- Bull, J.J. (1987) Temperature-sensitive periods of sex determination in lizard: similarities with turtles and crocodylians. *Journal of Experimental Zoology*, **241**, 143–148.
- Burnham, K.P. & Anderson, D.R. (1998) *Model Selection and Inference: A Practical Information – Theoretic Approach*. Springer, New York.
- Deeming, D.C. & Ferguson, M.W.J. (1989) Effects of incubation temperature on growth and development of embryos of *Alligator mississippiensis*. *Journal of Comparative Physiology – Part B*, **159**, 183–193.
- Desvages, G. & Pieau, C. (1992) Aromatase-activity in gonads of turtle embryos as a function of the incubation temperature of eggs. *Journal of Steroid Biochemistry and Molecular Biology*, **41**, 851–853.
- Dorizzi, M., Richard-Mercier, N., Desvages, G., Girondot, M. & Pieau, C. (1994) Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. *Differentiation*, **58**, 1–8.
- Ewert, M.A. & Legler, J.M. (1978) Hormonal induction of oviposition in turtles. *Herpetologica*, **34**, 314–318.
- Ewert, M.A., Jackson, D.R. & Nelson, C.E. (1994) Patterns of temperature-dependent sex determination in turtles. *Journal of Experimental Zoology*, **270**, 3–15.
- Georges, A. (1989) Female turtles from hot nests: is it duration of incubation or proportion of development as high temperatures that matters? *Oecologia*, **81**, 323–328.
- Georges, A., Beggs, K., Young, J.E. & Doody, J.S. (2005) Modelling development of reptile embryos under fluctuating temperature regimes. *Physiological and Biochemical Zoology*, **78**, 18–30.
- Georges, A., Doody, J.S., Beggs, K. & Young, J. (2004) Thermal models of TSD under laboratory and field conditions. *Temperature-dependent Sex Determination in Vertebrates* (eds N. Valenzuela & V. Lance). Smithsonian Books, Washington, DC.
- Georges, A., Limpus, C. & Stoutjesdijk, R. (1994) Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. *Journal of Experimental Zoology*, **270**, 432–444.
- Girondot, M. (1999) Statistical description of temperature-dependent sex determination using maximum likelihood. *Evolutionary Ecology Research* **1**, 479–486.
- Godfrey, M.H., Delmas, V. & Girondot, M. (2003) Assessment of patterns of temperature-dependent sex determination using maximum likelihood model selection. *Ecoscience*, **10**, 265–272.
- Gonzalez, A. & Piferrer, F. (2002) Characterization of aromatase activity in the sea bass: effects of temperature and different catalytic properties of brain and ovarian homogenates and microsomes. *Journal of Experimental Zoology*, **293**, 500–510.
- Jeyasuria, P. & Place, A.R. (1997) Temperature-dependent aromatase expression in developing diamondback terrapin (*Malaclemys terrapin*) embryos. *Journal of Steroid Biochemistry and Molecular Biology* **3**, 415–425.
- Lasdon, L.S., Waren, A.D., Jain, A. & Ratner, M. (1978) Design and testing of a generalized reduced gradient code for nonlinear programming. *ACM – Transactions on Mathematical Software* **4**, 34–50.
- Marcovaldi, M.A., Godfrey, M.H. & Mrosovsky, N. (1997) Estimating sex ratios of loggerhead turtles in Brazil from pivotal incubation durations. *Canadian Journal of Zoology*, **75**, 755–770.
- Mrosovsky, N. & Pieau, C. (1991) Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. *Amphibia – Reptilia*, **12**, 169–179.
- Pieau, C. (1982) Modalities of the action of temperature on sexual differentiation in field-developing embryos of the European pond turtle *Emys orbicularis* (Emyidae). *Journal of Experimental Zoology*, **220**, 353–360.
- Pieau, C. & Dorizzi, M. (1981) Determination of temperature sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys orbicularis*. *Journal of Morphology*, **170**, 373–382.
- Pieau, C. & Dorizzi, M. (2004) Oestrogens and temperature-dependent sex determination in reptiles: all in the gonads. *Journal of Endocrinology*, **181**, 367–377.
- Pieau, C., Dorizzi, M. & Richard-Mercier, N. (1999) Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cellular and Molecular Life Sciences*, **55**, 887–900.
- Pieau, C., Dorizzi, M., Richard-Mercier, N. & Desvages, G. (1998) Sexual differentiation of gonads as a function of temperature in the turtle *Emys orbicularis*: endocrine function, intersexuality and growth. *Journal of Experimental Zoology*, **281**, 400–408.
- R Development Core Team (2007) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.
- Raynaud, A. & Pieau, C. (1985) Embryonic development of the genital system. *Biology of the Reptilia* (eds C. Gans & F. Billett), pp. 149–300. John Wiley and Sons, New York.

- Sharpe, P.J.H. & DeMichelle, D.W. (1977) Reaction kinetics of poikilotherm development. *Journal of Theoretical Biology*, **64**, 649–670.
- Souza, R.R. & Vogt, R.C. (1994) Incubation temperature influences sex and hatchling size in the Neotropical turtle *Podocnemis unifilis*. *Journal of Herpetology*, **28**, 453–464.
- Vaillant, S., Dorizzi, M., Pieau, C. & Richard-Mercier, N. (2001) Sex reversal and aromatase in chicken. *Journal of Experimental Zoology*, **290**, 727–740.
- Valenzuela, N. (2001) Constant, shift, and natural temperature effects on sex determination in *Podocnemis expansa* turtles. *Ecology*, **82**, 3010–3024.
- Valenzuela, N., Botero, R. & Martinez, E. (1997) Field study of sex determination in *Podocnemis expansa* from Colombian Amazon. *Herpetologica*, **53**, 390–398.
- West, G.B., Brown, J.H. & Enquist, B.J. (2004) Growth models based on first principles or phenomenology? *Functional Ecology*, **18**, 188.
- Wibbels, T., Bull, J.J. & Crews, D. (1994) Temperature-dependent sex determination—a mechanistic approach. *Journal of Experimental Zoology*, **270**, 71–78.
- Yntema, C.L. (1968) A series of stages in the embryonic development of *Chelydra serpentina*. *Journal of Morphology*, **125**, 219–251.
- Yntema, C.L. (1976) Effects of incubation temperatures on sexual differentiation in the turtle, *Chelydra serpentina*. *Journal of Morphology*, **150**, 453–462.
- Yntema, C.L. & Mrosovsky, N. (1982) Critical periods and pivotal temperatures for sexual differentiation in loggerhead sea turtles. *Canadian Journal of Zoology*, **60**, 1012–1016.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1. R code (programming language version 2.5.0) of the mechanistic model of TSD

Fig. S1. Fluctuating temperature regimes inspired by records in natural nests of *E. orbicularis* and programmed in incubators.

Table S1. Model selection from a set of candidates based on the minimum Akaike's Information Criteria or AIC

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