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A mechanistic model of temperature-dependent sex determination in a Chelonian, the European pond turtle

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Running title: A mechanistic model of TSD in turtles

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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 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 mechanisms, 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.
52  *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 et al., 1985). To date, TSD is unknown in snakes and birds, but occurs in all studied crocodilians 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 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 et al., 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* (Linne, 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 thermosensitive period (TSP) that corresponds to the first stages of gonadal differentiation, these embryonic stages being approximately the same whatever TSD species studied (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 estrogens involvement in gonadal sex differentiation. The feminizing role of endogenous estrogens in the gonad has been shown during the TSP (Pieau et al., 2004). Some studies revealed that levels of gonadal estrogens are correlated with the gonadal activity of aromatase (i.e. the enzyme complex that converts androgens to estrogens) 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 et al., 1981; Deeming et al., 1989). Temperature also has a cumulative effect on aromatase activity, as well as 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).
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The European pond turtle, *Emys 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 to 22 of embryogenesis (Yntema, 1968) corresponding to an embryo mass between 170 and 1100 mg (Pieau et al., 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:

- 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 et al., 1981).
- The gonadal aromatase activity (fmoles/hours/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).
- The gonadal protein content (µmoles/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 and 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 modeled 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:
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 et al., 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 and 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). Unfortunately, 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 modeled as the product of two scaled Richards’ functions with equation (2):

$$m(t) = m_0 \exp \left( r_m \cdot t \right)$$

$$r_m = \frac{M_{\text{Max}}}{\left[ C + B \left( \exp \left( \frac{-\Delta X_m}{Y_m} \right) \cdot (X_m - T) \right) + \exp \left( \frac{1}{Y_m} \cdot (T - X_m - \Delta X_m) \right) \right]^{1/\alpha}}$$

This function included five parameters: $X_m$, $\Delta X_m$, $U_m$, $Y_m$ and $M_{\text{Max}}$. It modeled $r_m$ rising from 0 to the scaling parameter $M_{\text{Max}}$ and further declining from $M_{\text{Max}}$ to 0 as incubation temperature $T$ increases. We could impose an increasing growth rate between 20 and 35°C
and a decreasing growth rate above 35°C (C. Pieau, pers. obs.): the first inflexion point (between 0 to $M_{\text{Max}}$) is observed at incubation temperature $T = X_m$ and the second (between $M_{\text{Max}}$ to 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:

$$a(m) = a_0 \cdot \exp\left(r_a(m - m_{\text{esp}})\right)$$

(3)

where $a_0$ is the initial gonadal aromatase activity in eggs, $r_a$ 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 $(m - m_{\text{esp}})$, where $m_{\text{esp}}$ 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 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 estrogen 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 modeled as a scaled sigmoid logistic function (4):

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

(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 zero 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

\[
g(t) = g_0 \exp \left( r_g \cdot (t - t_{\text{tsp}}) \right)
\]

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 recalculated from equation (1). As gonads appear at the beginning of the TSP (i.e. \( t_{\text{tsp}} \)), we also performed an origin shift, represented here by \( (t - t_{\text{tsp}}) \), 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 estrogen 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 modeled as a classical logistic function (6):

\[
r_g = \frac{r_m}{1 + \exp \left( \frac{X_g - t_{Eq} - a}{Y_g} \right)}
\]
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 estrogen levels, represented by the equivalent aromatase growth rate $r_{Eq,a}$. $r_{Eq,a}$ is calculated from equation (4) using the embryo mass $m$ corresponding in raw data and the aromatase activity $a$ at the previous step time. 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 a maximum likelihood estimation with a Gaussian error. The mean value $m$ and the standard deviation $SD$ of each parameter were fitted. Using $\pm 2 \, SD$ 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 equations (3) and (5) for a temperature of 28.62°C (i.e. pivotal temperature that theoretically yield 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 equation (7), for each time step $\Delta t$ and without loss of precision:

$$y_{t+\Delta t} = y_t + y_t \cdot (\exp(r\Delta t) - 1)$$  \hspace{1cm} (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.

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 modeled. 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 – online) 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, B, C & D), inspired
by records in natural nests of *E. orbicularis* (Pieau, 1982), presented heterogeneous variations
of temperature (Fig. S1 - online). 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). Eggs were
placed in plastic boxes and ¾ buried in vermiculite with a controlled and intermediate water
potential (-398 Kpa: 0.44 g of sterilized water/g 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 weights 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 favor 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 simulations (i.e. 100 embryos) of our mechanistic model and estimated this proportion (from equation (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 kind of physiological processes, an exponential function was defined as the best model to represent the raw data (Table S1 - online). 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 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 $P = 28.62 \pm 3.74 \times 10^{-3}$ °C, a slope $S = -0.19 \pm 2.34 \times 10^{-3}$ and a range of temperatures that produce 5 to 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 to 30°C and a related thermal variance ranging from 0 to $5°C$. We verified that differential effect 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).

**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).
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 3,4), for a TSD Ia species, the European pond turtle (*Emys 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 fluctuation 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 *et al.*, 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 estrogen hormone pattern), all involved in the TSD mechanism (Fig. 1; Pieau et al., 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 et al., 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 weights. During the TSP, the gonad grows at the same time as the embryo and its sexual differentiation is controlled by estrogen 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
estrogen levels allocated by females to their eggs (i.e. parameter \(a_0\) in equation 3) or by initial
embryo mass (parameter \(m_0\) in equation 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 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 value of parameters from new fit on empirical data of the established TSD profiles.
Acknowledgements

We really thank Bernard Boussac, Suzette Bessède (“Tortues Passion”), Luc Fouveirol (“La ferme aux crocodiles”) and Didier Touzet (“ECATE”) for their warm welcome and their help to obtain gravid female slider turtles. We are grateful to Lionel Saunois and Annick Ambroise for their invaluable help during the collection of the eggs, as well as for their assistance in sexing juvenile turtles. Finally, we acknowledge Jean-Michel Guillon, Philippe Rivalan and Stephen Gregory for their suggestions and constructive comments on various drafts of the manuscript. We assure that this work has been performed in agreement with the current laws for experimentation in France.
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Supplementary material

The following supplementary material is available for this article online from http://blackwell-synergy.com.

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.
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 hours-sinusoidal with a constant range of temperature, while in 2004, temperature regimes were fluctuating with heterogeneous variations in temperature (Fig. S1 - online).

<table>
<thead>
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<tbody>
<tr>
<td></td>
<td>26 ± 3.5°C</td>
<td>28 ± 0.9°C</td>
<td>27 ± 2.6°C</td>
<td>29 ± 3°C</td>
<td>28.5 ± 5°C</td>
<td>28.5 ± 1.5°C</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<tr>
<td>Males</td>
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<td>14</td>
<td>18</td>
<td>3</td>
<td>2</td>
<td>21</td>
<td>1</td>
<td>0</td>
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</tr>
<tr>
<td>Females</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>29</td>
<td>11</td>
<td>21</td>
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<td>22</td>
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<tr>
<td>$SR_f$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.91</td>
<td>0.93</td>
<td>0.34</td>
<td>0.95</td>
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Table 2. Summary of models’ simulations and predictions of sex-ratio (SR_f), expressed as the frequency of females, for two kind 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.

<table>
<thead>
<tr>
<th>Regime</th>
<th>Thermal variance</th>
<th>Mechanistic model</th>
<th>CTE model</th>
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<tbody>
<tr>
<td></td>
<td>Dev. Prop.</td>
<td>Predicted SR_f</td>
<td>CTE</td>
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<tr>
<td>26 ± 3.5°C</td>
<td>constant</td>
<td>0.467</td>
<td>27.52</td>
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<tr>
<td>28 ± 0.9°C</td>
<td>constant</td>
<td>0.364</td>
<td>28.09</td>
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<td>27 ± 2.6°C</td>
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<td>29 ± 3°C</td>
<td>constant</td>
<td>0.685</td>
<td>29.90</td>
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<tr>
<td>28.5 ± 5°C</td>
<td>constant</td>
<td>0.553</td>
<td>30.83</td>
</tr>
<tr>
<td>28.5 ± 1.5°C</td>
<td>constant</td>
<td>0.429</td>
<td>28.76</td>
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<td>B</td>
<td>heterogeneous</td>
<td>0.483</td>
<td>-</td>
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<td>C</td>
<td>heterogeneous</td>
<td>0.542</td>
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<td>D</td>
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<td>0.488</td>
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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 for T < 27.86 °C
- SR_f = 0.678 T – 18.896 for 27.86 < T < 29.33 °C (R^2 = 0.89, n = 15)
- SR_f = 1 for T > 29.33°C
**Figure legends**

**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.

**Fig. 2.** Simulation procedure of the physiological processes modeled 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.

**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 ± SE of the pivotal temperature P, the slope of the function S and the transitional range of temperatures TRT_{95%} are specified.

**Fig. 4.** Observed versus 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.
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-hours sinusoidal temperature regime.
Fig. 1.
Fig. 2
Fig. 3.
Fig. 4.
Fig. 5.