

Modeling Thermal Influence on Animal Growth and Sex Determination in Reptiles: Being Closer to the Target Gives New Views

M. Girondot^{a, b} S. Ben Hassine^{a-c} C. Sellos^{a, b} M. Godfrey^d J.-M. Guillon^{a, b}

^aLaboratoire Écologie, Systématique et Évolution (UMR8079), Faculté des Sciences d'Orsay, Université Paris-Sud, ^bAgroParisTech, CNRS, Orsay, France; ^cDépartement des Sciences de la Vie, Unité Biodiversité et Écosystèmes Aquatiques, UR05ES05, Faculté des Sciences de Sfax, Sfax, Tunisie; ^dNorth Carolina Wildlife Resources Commission, Beaufort, N.C., USA

Key Words

Aromatase · Estrogen · Estradiol · Reptile · Sex determination · Sex differentiation

Abstract

Many species of oviparous reptiles, including crocodylians, a majority of turtles, some lizards and the 2 closely related species of *Sphenodon* have been shown to display temperature-dependent sex determination (TSD). Whereas it has been demonstrated very early that TSD also occurs in natural conditions, the relationship between a time series of changing temperatures and sex ratio remains a challenging problem for reptiles. We describe how a physiological model of embryo growth, gonadal development and aromatase activity can produce outputs that mimic well TSD. We provide an enhancement of a previously published model taking into account direct effect of temperature on aromatase activity. The comparison between the original model and the new one suggests that aromatase expression is controlled by a repressor factor expressed at masculinizing temperatures rather than its enhancement at feminizing temperatures.

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Many species of oviparous reptiles, including crocodylians, a majority of turtles, some lizards and the 2 closely related species of *Sphenodon* have been shown to display temperature-dependent sex determination (TSD). In these species, the differentiation of gonads into ovaries or testes depends on the incubation temperature of the embryo during a critical period of embryonic development designated the thermosensitive period (TSP) [Yntema and Mrosovsky, 1982; Mrosovsky and Pieau, 1991]. This period begins with the appearance of the gonad during embryogenesis and encompasses the middle third of embryo development. It approximately underlies the same embryonic stages whatever TSD species [Pieau and Dorizzi, 1981; Ferguson and Joanen, 1983; Bull, 1987; Webb et al., 1987; Lang and Andrews, 1994].

TSD mechanism was formerly described by defining different patterns, which correlate sex ratio and constant incubation temperatures in laboratory conditions [Ewert et al., 1994]. Three patterns have been described: (i) MF, (ii) FM and (iii) FMF. In the MF pattern, lower incubation temperatures are masculinizing whereas higher ones are feminizing; the reverse is true for the FM pattern. In the FMF pattern, both the lower and the highest temperatures are feminizing whereas the intermediate ones are masculinizing. Both sexes are produced in variable proportions within a range of intermediate temperatures,

called the transitional range of temperature (TRT). At the theoretical 'pivotal temperature' P , 50% of each sex is yielded [Mrosovsky and Pieau, 1991].

Most of the data about relationship between temperature and sex determination in reptiles with TSD has been obtained from artificial incubation at constant temperatures. Whereas it has been demonstrated long time ago that TSD occurs also in natural conditions [Pieau, 1974b], the relationship between a time series of changing temperatures and sex ratio has been rarely investigated. Georges and colleagues have proposed different models to take into account temperature fluctuations during incubation in sex ratio predictions [Georges, 1989; Georges et al., 1994, 2004]. The more advanced ones use degree-hour model and constant-temperature equivalent statistic (CTE) to convert fluctuating temperature into constant temperature that would have produced the same effect. Following this idea, Delmas et al. [2008] integrate the actual knowledge of physiological response of the embryo to temperature and show that both the sex ratio at constant and fluctuating temperatures can be explained. In this review, we will discuss the various approaches authors have used to investigate the effect of fluctuating temperature on sex ratio and discuss the way by which temperature effects on growth of embryos and gonads and on sex determination can be modeled. Delmas et al. [2008] recognize that 'a more realistic model (of aromatase activity) might be one similar to the model of embryo growth rate with an inhibition effect of too high temperatures'. We present such an enhancement here and discuss the pattern of regulation of aromatase in the light of recently published gene expression data (see reviews in this issue).

Modeling the Effect of Temperature on Sex Ratio

Constant Temperatures and Sex Ratio

Whereas it is not the main interest of this review, it seems necessary to describe first the effect of constant temperature on sex ratio. The relationship has been fitted successively using a logistic equation [Girondot, 1999] for pattern MF or FM or the product of 2 modified logistic equations to render FMF pattern [Godfrey et al., 2003]. Recently, we expanded this work to all the species studied so far and we search particularly to estimate if asymmetrical response of sex ratio around pivotal temperature (P) is observed (i.e. is 1°C below P identical to 1°C above?). In 7 out of the 29 species for which this effect has been tested, an asymmetry model was selected [Hulin et al., 2009].

Interestingly, the best studied species exhibit asymmetry which could indicate that lack of detection in others is due to lack of power in the test.

The asymmetry indicates that the relative effect of 1°C change on sex ratio is not the same depending on the initial temperature. This observation confirms that complex interplay between sex and temperature occurs.

Fluctuating Temperatures and Sex Ratio (table 1)

The knowledge of P is not enough to predict the sex ratios in natural conditions when incubation temperatures during the TSP fluctuate around P . The first proxy of incubation temperature being used to predict the sex ratio under fluctuating temperatures was the mean temperature during the estimated middle third of the incubation period. As expected from results at constant temperatures, a trend was generally observed for MF turtles, i.e. a higher mean temperature increased the proportion of females produced. Predictions based on the comparison between P and the mean incubation temperature, or based on the amount of time spent above P during the TSP also found some support from field data. However, studies that searched for a simple relationship between the mean temperature (or the time spent above P) and the sex ratio often found that a large proportion of the variance remained unexplained [Bull, 1985; Schwarzkopf and Brooks, 1987; Demuth, 2001; Godley et al., 2001; Valenzuela, 2001; San et al., 2004].

The Degree-Hour and the Constant-Equivalent Temperature

Thus, results of early field studies were in broad agreement with laboratory studies, but it was soon observed that mean incubation temperature was not the only factor involved, and that variance was also important [Pieau, 1982; Bull, 1985]. Then it has been proposed that the outcome of sexual differentiation depends more on the relative proportion of embryo development taking place above and below the pivotal temperature than on the relative time spent above and below the pivotal temperature [Pieau, 1982]. This idea has been successfully tested using incubations with varying thermal variance but same mean temperature [Georges, 1989].

Georges [1989] proposed that under fluctuating temperature regimes, female turtles will be produced if more than half of embryonic development occurs at temperatures above the pivotal temperature each day, and males will be produced if more than half of daily embryonic development occurs below the pivotal temperature. The daily temperature above and below at which half of de-

Table 1. Proxies of fluctuating temperature effects on sex determination and sex ratios in turtles. Studies were included only if the temperature was recorded at least twice daily (at minimum and maximum) or once daily when diurnal variation of temperature was found to be negligible

Proxy	Evidence for the proxy being tested	Observation/experiment	Species and references
Mean temperature ^a	Qualitative ^d	Natural nests	<i>Caretta caretta</i> : Maxwell et al. [1988]; <i>Chelonia mydas</i> : Broderick et al. [2000]
		Natural and artificial nests	<i>Podocnemis expansa</i> : Alho et al. [1985] ^f
		Natural and artificial nests, Styrofoam boxes	<i>Lepidochelys olivacea</i> : Standora and Spotila [1985]
		Artificial nests and Styrofoam boxes	<i>Chelonia mydas</i> : San et al. [2004]
	Styrofoam boxes	<i>Dermochelys coriacea</i> : Dutton et al. [1985]	
Quantitative ^e	Laboratory incubator	<i>Graptemys ouachitensis</i> , <i>Graptemys pseudogeographica</i> : Bull and Vogt [1979]	
	Natural nests	<i>Caretta caretta</i> : Kaska et al. [1998], Öz et al. [2004], Kaska et al. [2006]; <i>Chelonia mydas</i> : Kaska et al. [1998], Horikoshi [1991], Godley et al. [2002]; <i>Chelydra serpentina</i> : Kolbe and Janzen [2002]; <i>Chrysemys picta</i> : Schwarzkopf and Brooks [1985] ^g ; <i>Graptemys</i> sp.: Bull [1985]	
No evidence	Natural and artificial nests	<i>Podocnemis expansa</i> : Valenzuela [2001] ^g ; <i>Podocnemis unifilis</i> : De Souza and Vogt [1994]	
	Natural nests	<i>Chrysemys picta</i> : Schwarzkopf and Brooks [1985]; <i>Gopherus polyphemus</i> : Demuth [2001]	
Artificial nests	<i>Chelonia mydas</i> : Leh et al. [1985]		
Thermal score ^b	Qualitative ^d	Natural nests	<i>Carettochelys insculpta</i> : Georges [1992]
Mean temperature compared to P^a	Qualitative ^d	Natural nests	<i>Dermochelys coriacea</i> : Binckley et al. [1998]
	No evidence	Artificial nests	<i>Lepidochelys olivacea</i> : Martinez and Páez [2000]
Mean of temperatures above P^a	Quantitative ^e	Natural and artificial nests	<i>Podocnemis expansa</i> : Valenzuela et al. [1997]
Time spent above P^a	Qualitative ^d	Natural nests	<i>Dermochelys coriacea</i> : Rimblot-Baly [1986–1987]
		Natural and artificial nests	<i>Podocnemis expansa</i> : Valenzuela et al. [1997]
		Artificial nests	<i>Chelydra serpentina</i> : Wilhoft et al. [1983]; <i>Emys orbicularis</i> : Pieau [1982]
	Quantitative ^e	Laboratory incubator	<i>Emys orbicularis</i> : Pieau [1973]
		Natural nests	<i>Chrysemys picta</i> : Schwarzkopf and Brooks [1985]; <i>Graptemys</i> sp.: Bull [1985]
Natural and artificial nests	<i>Chrysemys picta</i> : Weisrock and Janzen [1999] ^h ; <i>Podocnemis unifilis</i> : De Souza and Vogt [1994]		
Variance of temperatures ^a	Qualitative ^d	Artificial nests	<i>Emys orbicularis</i> : Pieau [1982]
	Quantitative ^e	Natural nests	<i>Graptemys</i> sp.: Bull [1985]
		Natural and artificial nests	<i>Podocnemis unifilis</i> : De Souza and Vogt [1994]
Mean daily CTE ^{a, c}	Qualitative ^d	Natural nests	<i>Carettochelys insculpta</i> : Doody et al. [2004]; <i>Emys orbicularis</i> : Georges [1989] ⁱ ; <i>Gopherus polyphemus</i> : Demuth [2001]
	Quantitative ^e	Natural nests	<i>Graptemys</i> sp.: Georges [1989] ^j
		Laboratory incubator	<i>Caretta caretta</i> : Georges et al. [1994] ^k ; <i>Emys orbicularis</i> : Delmas et al. [2008] ^k
	No evidence	Natural and artificial nests	<i>Podocnemis unifilis</i> : De Souza and Vogt [1994] ^l
Mechanistic model	Quantitative ^e	Laboratory incubator	<i>Emys orbicularis</i> : Delmas et al. [2008] ^k

^a During the estimated TSP (usually the middle third of incubation period). ^b Defined as the mid-point between daily maximum and minimum temperatures, estimated at 5 days in the estimated TSP. ^c CTE: Constant-temperature-equivalent (see text). ^d The conclusion was reached that higher values of parameter tend to give females; lower values tend to give males. ^e A statistically significant positive relationship (or positive effect) was

found with (on) the sex ratio. ^f Mean temperature was calculated over the whole incubation period. ^g The mean was calculated as °C × time spent above the threshold temperature for survival. ^h Marginally significant relationship. ⁱ Data from Pieau [1982]. ^j Data from Bull [1985]. ^k No statistically significant difference was found between the observed sex ratios and the predictions of the model. ^l Result discussed in Georges et al. [2004].

velopment occurs is calculated as a constant-temperature equivalent (CTE) [Georges et al., 1994]. A temperature regime that fluctuates will be equivalent, in terms of hatchling sex ratios, to a constant-temperature incubator set at the value of the CTE [Georges et al., 1994]. Various ways could be used to integrate the arbitrary general regime of fluctuating temperatures into a CTE, but in any case a model of developmental rate upon temperature is necessary [Georges et al., 2004] and these authors propose to estimate the CTE on a daily basis during the thermosensitive period of development (TSP). When CTE consistently exceeds pivotal temperature or is below, sex could be inferred safely, but when daily CTEs move through or oscillate about pivotal temperature during the TSP, the interpretation for sex ratio is more difficult and is not quantitative [Georges et al., 2004; Delmas et al., 2008].

A Physiological Model for Reptile Development with TSD

Following the idea that understanding TSD under fluctuating regime requires a model of growth of the embryo, Delmas et al. [2008] integrate the actual knowledge of physiological response of the embryo to temperature change into a single framework to decipher the effect of temperature on sex determination.

Embryo Growth Model

Ample information indicates that higher incubation temperatures shorten the duration of embryo development in reptiles [e.g. Miller, 1985]. The relationship between temperature and duration of embryo development follows a negative asymmetrical bell-shape curve (i.e. peak is shifted to the right of the curve) with developmental rate being null at low and high temperature and highest at some intermediate temperature [Georges et al., 2004]. The complete shape often is not known because lower and higher temperatures can reach the limits of lethality for the studied species. Various equations meet the general shape of developmental rate upon temperature [Sharpe and DeMichelle, 1977; Schoolfield et al., 1981; Zwietering et al., 1991; Alber and Schaffner, 1993; Delmas et al., 2008]. These models can be distinguished only when a very large number of observations are available, which is generally not the case for reptile development.

The development of reptile embryos has been assessed by the measurement of a variety of characters including lengths (total, head, flippers, tail, plastron, carapace or bridge for turtles for example) or mass. The advantage of

using mass is that it is measurable during all the development contrary to the various lengths which are generally reliable only during part of development [Miller, 1985].

The growth of animals is generally modeled with Gompertz [1825], von Bertalanffy [1938] or Richards [1959] differential equations. When δt is small, a simple recurrent equation $y_{t+\delta t} = y_t + \delta y_t \rightarrow_{t+\delta t}$ produces satisfactory results [Press et al., 1992]. On the other hand, when δt becomes large, the Runge-Kutta method is generally used to approximate solutions of ordinary differential equations [Atkinson, 1989], but this method is time-consuming. The beginning of all these models of animal growth is exponential with a general form being: $y_t = ae^{rt}$. The change of embryo mass slows down only during the days before hatching, but this phenomenon occurs after the TSP, which is out of the window of interest when TSD is modeled.

Contrary to more complex models, the exponential growth model permits an easy mathematical integration to model change of temperature during development without loss of precision or time-consuming correction. The recurrence relationship becomes: $y_{t+\delta t} = y_t + y_t(e^{r\delta t} - 1)$ and $y_0 = a$ (note a typographical error in equation 7 of Delmas et al. [2008] that is corrected here).

The use of embryological stages greatly facilitates discussion about development. Embryological stages are defined on a species or a group of species level based on key features of development. During early development, stages and mass of embryo are strongly correlated [Pieau and Dorizzi, 1981]. The correlation is lowered as the embryonic development reaches its end [Girondot, personal observ.].

Gonadal Growth

Little direct information is available to model the growth of the gonad. However, the growth of organs of the turtle *Chelydra serpentina* has been measured in dry and wet substrates that are known to induce differences in hatchling size. Scaled masses of liver, stomach, lungs, kidneys, and small intestine did not differ between turtles emerging in wet environments and those hatching in dry environments. Only, hearts of turtles hatching in dry settings were substantially larger than those of animals hatching in wet ones. Thus, the mass of most organs in turtles scaled to body size [Packard et al., 2000]. The gonad is bipotent when it appears and during the first stage of its development, and it acquires progressively its sexual characteristics. Gonadal growth has been estimated in *Emys orbicularis* by measuring the protein content of the entire organ at different stages of embryonic development, from

the beginning of TSP to hatching. Ovaries grow more slowly than testes during the first stages of gonadal differentiation, mainly during TSP [Pieau et al., 1998]. After TSP, growth of the ovary increases strongly to reach testis protein content just before hatching [Pieau et al., 1998].

Several studies using treatments with exogenous estrogens, anti-estrogens or aromatase inhibitors at male and female-producing temperatures have shown the key role of estrogens (i.e. feminizing hormones) in sexual differentiation of the gonad during TSP [Dorizzi et al., 1994; Richard-Mercier et al., 1995]. Thus, the growth of the gonads can be modeled using 2 additive components: its rate is similar to growth rate of the containing embryo but is also inhibited by the amount of estrogen in the gonad.

Estrogens and Aromatase Activity in the Gonad

The enzyme cytochrome P-450 aromatase converts testosterone and androstenedione to estrogens estradiol-17 β and estrone respectively. High levels of estradiol-17 β and estrone in the gonad block the differentiation of testicular cords in medulla and enhance the proliferation of the germinal epithelium to differentiate into an ovary. Strong similarities exist between aromatase activity and estrogen content in the gonad during TSP, leading to the assumption that aromatase activity is a proxy of estrogen content in the gonad [Dorizzi et al., 1991]. The aromatase activity measured in the gonad was shown to be positively related to incubation temperature during TSP [Desvages and Pieau, 1991, 1992; Desvages et al., 1993]. At the beginning of TSP, aromatase activity (i.e. estrogen level) is very low both at male- and female-producing temperatures. It remains low in the future testis all over TSP, while it increases exponentially in the future ovary [Desvages and Pieau, 1992].

In *Alligator mississippiensis*, aromatase assays carried out at 30°C and at 33°C produced similar rates, and Smith et al. [1995] concluded that ‘aromatase activity is not thermosensitive’. This conclusion is perhaps a little bit speculative, as it is known that any biochemical activity is sensitive to temperature from Arrhenius’ empirical equation and Eyring’s theoretical equation [Glasstone et al., 1941]. A more acceptable conclusion will be that given the small difference between the 2 assay temperatures, no significant difference has been detected. Surprisingly, we do not find more complete assays of aromatase sensitivity to temperature in reptiles, whereas it has been suspected very early that aromatization was a key process to explain TSD [Pieau, 1974a].

Aromatase sensitivity to assay temperature has been published for the 2 isoforms of aromatase in sea bass

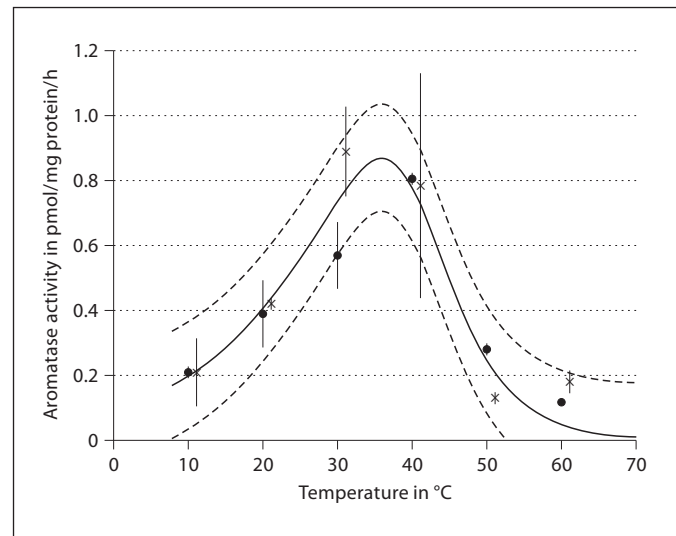


Fig. 1. Sea bass aromatase activity upon temperature of assay (•, Cyp19a, ovary isoform; ×, Cyp19b, brain isoform) modified from Gonzalez and Piferrer [2002]. The bars represent twice the standard deviations. The central curve is the fitted Schoolfield equation [Schoolfield et al., 1981] using maximum likelihood with gaussian link and the envelope is twice the fitted standard deviation.

[Gonzalez and Piferrer, 2002]. These 2 isoforms (*cyp19a* and *cyp19b*) originated from an ancient duplication in teleosts. *Cyp19a* is expressed mainly in the follicular cells lining the vitellogenic oocytes in the ovary during vitellogenesis. *Cyp19b* is expressed abundantly in the brain, at the hypothalamus and ventral telencephalon extending to the olfactory bulbs [Chiang et al., 2001]. Disruption of *cyp19a* produces masculinization in zebrafish [Fenske and Segner, 2004]. The date of duplication has not been established so far, but from figure 2 in Tong et al. [2001], it could be dated before the divergence of Mammalia and Sauropsidia, 310 millions years ago. The rate of aromatization for the 2 isoforms of sea bass aromatase upon temperature has been obtained from figure 1B and C in Gonzalez and Piferrer [2002] and the data have been fitted using the Schoolfield equation [Schoolfield et al., 1981] using maximum likelihood and gaussian distribution. To test whether both isoforms have evolved different temperature sensitivity after their duplication, a single model is also fitted on both enzymes. Using AIC model selection [Burnham and Anderson, 2002], we show that the single model is sufficient to explain the effect of temperature on both isoforms (isoform-specific models: AIC = -31.93; single model: AIC = -32.83; fig. 1). Furthermore, the zebrafish aromatase isoforms have kinetic properties

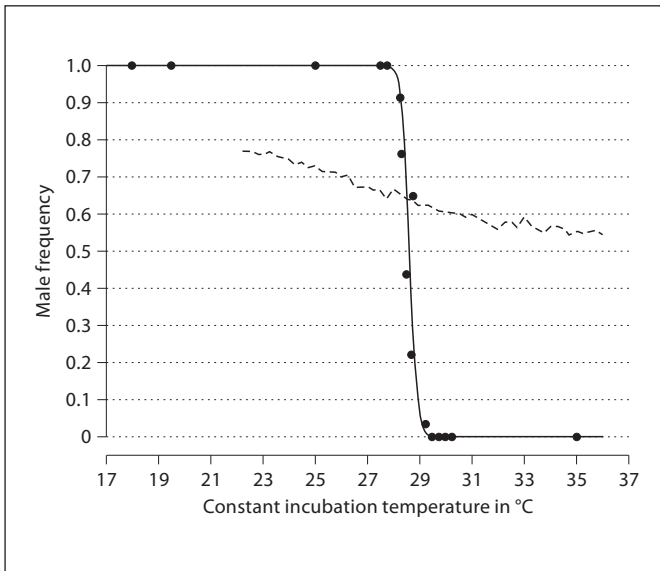


Fig. 2. Observed (●) [reviewed in Girondot, 1999] and modeled sex ratio (continuous line: Delmas et al. [2008]; dashed line: this paper) dependent on constant incubation temperature.

similar to the mammalian P450 aromatase [Tong et al., 2001]. Thus, we postulate that the aromatase enzyme activity upon temperature measured in sea bass is conserved between teleosts and amniotes and can be used as a reference for reptiles. Obviously, it will be interesting to test this hypothesis in future.

In order to test whether the aromatase enzyme activity dependent on temperature could depict the pattern of TSD in reptiles, we replace the fitted function for the regulation of aromatase in the published mechanistic model for TSD in the turtle *Emys orbicularis* [equation 4 in Delmas et al., 2008] with the new one obtained here (fig. 1). The scale of the aromatase activity rate as well as all other parameters that describe embryo and gonadal growth rate have been adjusted as the set of parameters that best describe the embryo and gonadal mass, the aromatase activity upon developmental time and temperature [see Delmas et al., 2008 for details of fitting procedure].

Whereas the original model fits well the sex ratio both at constant and fluctuating temperature, the modified one performs very poorly (fig. 2). This can be easily understood when comparing the original and newly fitted aromatase activity upon temperature (fig. 3, curves A and B). The newly fitted aromatase activity is still high when masculinizing temperatures are reached. To com-

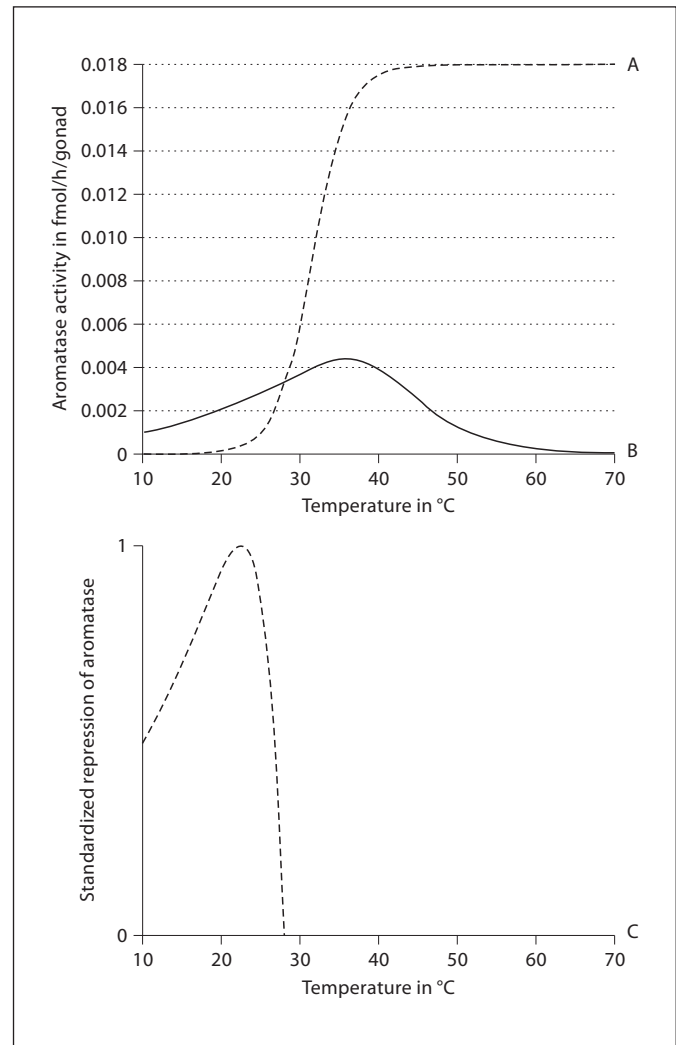


Fig. 3. Fitted model of aromatase activity change upon temperature that includes both the enzyme activity dependent on temperature and the transcriptional regulation (A) or only the enzyme activity dependent on temperature (B). The transcriptional repression (C) is obtained by standardized difference of curves B and A.

pensate this effect during the fitting procedure, the fitted scale is much lower than for the previous curve. Thus, females are still produced at low temperatures and males are still produced at higher temperatures (fig. 2). Nevertheless, only taking into account the sensitivity of aromatase enzyme to temperature, we are able to model a dependency of sex ratio to temperature. Such a model could explain the thermosensitivity of sex determination in sea bass [Blazquez et al., 1998; Koumoundouros et al., 2002], the species from which the aromatase enzyme activity

rates used here were obtained. Furthermore, this model that just includes enzymatic response to temperature could be the origin of TSD in many species as well as in reptiles [Georges et al., 2009].

The original model [Delmas et al., 2008] aggregates 2 levels of regulation of aromatase: the temperature effect on the aromatase activity as described newly here but also a putative transcriptional regulation. The difference between these 2 models (fig. 3, curves A and B) can be interpreted as the effect of regulation of transcription of aromatase (fig. 3, curve C) and consequently a negative transcriptional regulator of aromatase can be proposed to act at lower temperature (i.e. masculinizing) rather than an activation of aromatase at higher temperature. This hypothesis could parsimoniously explain the pattern FMF as resulting from a constitutive expression of aromatase and the activation of a repressor at masculinizing temperatures rather than 2 activations of aromatase gene expressions.

Discussion

The origin, evolution and maintenance of sensitivity of sex determination to temperature in reptiles have been discussed in nearly half of the published papers about TSD. Various models have been proposed [Valenzuela, 2004] and few tests have been performed [Janzen, 1995; Warner and Shine, 2005, 2008]. The large taxonomic diversity of species with TSD and the large life-history variation for these species make any general conclusion about the evolution of TSD difficult. Furthermore, most of the data about the relationship between temperature and sex determination have been obtained from artificial incubation at constant temperature. Whereas it has been demonstrated very early that TSD also occurs in natural conditions [Pieau, 1974b], the relationship between a time series of changing temperatures and sex ratio remains a challenging problem for reptiles. This gap in knowledge clearly does not help to understand the origin, evolution and maintenance of TSD.

Georges and colleagues have proposed different models to take into account temperature fluctuations during incubation in sex ratio predictions [Georges, 1989; Georges et al., 1994, 2004]. The more advanced ones use degree-hour model and constant-temperature equivalent statistic (CTE) to convert fluctuating temperature into a constant one that would have produced the same effect. Following this idea, Delmas et al. [2008] integrate the actual knowledge of physiological response of embryo to

temperature and show that both the sex ratio at constant and fluctuating temperatures can be accurately explained. We here confirm that aromatase enzyme activity cannot by itself explain the TSD mechanism but is important enough to be taken into account. We postulate that a negative regulator of aromatase gene expression is expressed at male-producing temperatures. Both *SOX9* and *DMRT1* could play this role [Shoemaker et al., 2007]. The regulation of aromatase transcription by estradiol could be an important factor to be included in the model. Data from reptiles are lacking, but this hypothesis has been tested in fishes. In zebrafish, a slight upregulation of aromatase by estradiol has been observed in vitro [Cheshenko et al., 2007]. In the fish fathead minnow (*Pimephales promelas*), model synthetic estrogen 17 α -ethinylestradiol (EE2) increased the expression of aromatase genes (*cyp19a* and *cyp19b*) and decreased the expression of genes involved in testis differentiation (anti-Müllerian hormone (*amh*) and mab-3-related transcription factor 1 (*dmrt1*) [Filby et al., 2007]). Eleven promoters have been found so far for the human aromatase gene and for most of them the pattern of regulation is not known [Demura et al., 2008]. Furthermore, this complex pattern of expression was only studied in mammals which are known to have a different pattern of regulation as compared with vertebrates sensitive to estrogen for gonadal sex determination [Barske and Capel, 2008]. Further studies in reptiles with TSD are needed to add new data for a more complete mechanistic model of TSD.

In the current version of the model, all the variability is introduced as characteristics of embryo whereas environmental variation should be also included. For example, embryos at the top of the nest experience a larger variance of temperatures [Wilhoft et al., 1983; Maxwell et al., 1988; Georges, 1992; Demuth, 2001] or higher temperatures [Wilhoft et al., 1983; Maxwell et al., 1988; Kaska et al., 1998, 2006]. In both cases, the CTE is higher at the top of the nest and feminization occurs [Demuth, 2001] or is expected. Another source of variation could result from a lack of information about the exact timing and duration of the thermosensitive period (TSP) during development at fluctuating temperatures [Georges et al., 2004]. If a temperature trend is observed during development, the TSP will not be located at the middle third of development but shifted at the beginning or the end according to direction of temperature change.

The model shown here can be applied only to species that have been extensively studied. An alternative will be to use the same set of equations and to fit the parameters

adjusting best the sex ratios produced with known time series of temperatures. Such a fit has been already performed for *Trachemys scripta elegans* at constant temperatures [Delmas, 2006] but is still under development for fluctuating temperatures.

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