Genetic contribution to sex determination in turtles with environmental sex determination

MARC GIRONDOT[1]*, PATRICK ZABORSKI[1,2], JEAN SERVAN[2] AND CLAUDE PIEAU[1]

[1] *Laboratoire de Biochimie du Développement, Institut Jacques Monod, Centre National de la Recherche Scientifique et Université Paris 7, 2 place Jussieu, 75251 Paris CEDEX 05, France*
[2] *Centre de Biologie Cellulaire, Centre National de la Recherche Scientifique, 67 rue Maurice Günsbourg, 94205 Ivry-sur-Seine CEDEX, France*
[3] *Laboratoire d'Évolution des Systèmes Naturels et Modifiés, Muséum National d'Histoire Naturelle, 36, rue Geoffroy-Saint-Hilaire, 75005 Paris, France*

Summary

In many reptiles, sex determination is temperature-sensitive. This phenomenon has been shown to take place in the laboratory as well as in nature, but its effect on natural populations remains questionable. In the turtle *Emys orbicularis*, the effects of temperature override a weak mechanism of genetic sex determination which is revealed in incubation at pivotal temperature. At this temperature, the sexual phenotype is concordant with the expression of the serologically defined H-Y antigen (H-Ys) in non-gonadal tissues; males are H-Ys negative (H-Y⁻) whereas females are H-Ys positive (H-Y⁺). To estimate the importance of sexual inversion (sexual phenotype and H-Ys expression discordant) in populations of Brenne (France), the frequencies of male and female sexual phenotypes among H-Ys phenotypes were determined. The frequencies of sex reversed individuals are low, only 6% of phenotypic females being H-Y⁻ and 11% of phenotypic males being H-Y⁺. According to these data, two theoretical models have been constructed to estimate the contribution to sex determination of individuals in relation to their genotype. The first model excludes any influence of incubation temperature and sexual phenotype on the fitness of individuals. The second one considers that these parameters influence fitness because this model has been previously shown to favour environmental sex determination. In both models, it appears that sex determination can be viewed as genotypic and monogenic with some individuals sexually inverted by the action of temperature. One category of homozygous animals differentiates mainly into one sex, and the heterozygous animals differentiate mainly into the other sex. The second category of homozygotes has a low frequency in the populations and can differentiate as male or female without high constraint. Then it is estimated that in Brenne approximately 83% of the eggs are incubated in conditions allowing the genetic component to influence sex determination.

1. Introduction

In many reptile species, sex determination is temperature-dependent (TSD: reviewed by Bull, 1980; Raynaud & Pieau, 1985; Janzen & Paukstis, 1991*a*). A theory considering that ESD (environmental sex determination) is adaptive has been proposed by Charnov & Bull (1977). In this theory, ESD evolves when: (i) the environment that offspring experience affects the fitness of each sex differently; (ii) the environment that offspring enter cannot be chosen; and (iii) offspring from different environments mate with one another (Charnov & Bull, 1977). This theory agrees with the occurrence of ESD in some species, for example in the fish *Menidia menidia* (Conover, 1984). Hypotheses based on potential growth rate and adult sexual dimorphism have been proposed for the advantage of ESD in reptiles (Deeming & Ferguson, 1989; Ewert & Nelson, 1991), but they are still open to discussion and remain to be demonstrated (Bull & Charnov, 1989; Janzen & Paukstis, 1991*b*). In the Alligator, a mathematical model takes into account age distributions to show that populations can be maintained with ESD but will become extinct with GSD (Genotypic sex determination) (Woodward & Murray, 1993).

* Corresponding author.

A genetic component in sex determination has been described for some reptile species with TSD. Morphologically differentiated sex chromosomes have been shown in *Staurotypus salvinii* (Sites *et al.* 1979), a turtle species whose sex determination appears to be sensitive to temperature at least in one population (Ewert & Nelson, 1991). The same situation exists in the lizard *Gekko japonicus* (Yoshida & Msahiro, 1974 cited in Deeming and Ferguson, 1991; Tokunaga, 1985). A sex-specific DNA has been found in the males of the marine turtles *Chelonia mydas* and *Lepidochelys kempii* by hybridization with Bkm, a probe containing mainly tandem repeats of GATA and GACA (Demas *et al.* 1990). These two species display temperature-dependent sex determination (TSD). Moreover, in *Chelonia mydas*, Wellins (1987) showed that the level of expression of the serologically defined H-Y antigen was high in males and low in females.

The H-Y antigen was originally defined as a male-specific minor histocompatibility antigen in the mouse (Eichwald & Silmser, 1955). So far, only immunological techniques permit the detection of H-Y antigen and, according to the method used, three H-Y antigens may be defined (reviewed by Wiberg, 1987): (i) H-Yt, detected by transplantation experiments, (ii) H-Yc, detected by cytotoxic T-lymphocytes, and (iii) H-Ys, typed by serological techniques using anti-H-Y antibodies.

Although the gene encoding or controlling H-Yt was grossly located on the Y chromosome, that of H-Yc was mapped to the short arm of the mouse Y chromosome (McLaren *et al.* 1984), and to the long arm of the human Y chromosome (Cantrell *et al.* 1992), and is distinct from the testis-determining gene (mouse *Tdy*, human *TDF*). The most promising candidate for *Tdy* (and *TDF*) is currently the *Sry* gene (*SRY* for human) (Gubbay *et al.* 1990; Sinclair *et al.* 1990).

H-Yc could play a role in spermatogenesis (Burgoyne *et al.* 1986). H-Ys has not been mapped, but according to various data, it has been proposed that the structural gene is autosomal and the regulatory one is on the Y chromosome (Wolf, 1985). The original proposal that H-Ys is the primary testis-determining factor (Wachtel *et al.* 1975) is now abandoned since Goldberg *et al.* (1991) demonstrated the contrary, using sex-reversed mice, and also suggested that H-Ys could be a factor required for spermatogenesis in the mouse.

Nevertheless, H-Ys is a male-specific factor in mammals, and is also ubiquitously conserved in many non-mammalian vertebrates including fish, amphibians, birds, and reptiles (Wachtel, 1983; Nakamura *et al.* 1987). Moreover, as the level of its expression is higher in the heterogametic sex, except for very few species, H-Ys can be considered as a marker of the heterogametic sex. Non-mammalian species are well known for the possible action of epigenetic factors in

the process of gonadal differentiation. By reinvestigating such experiments it has been shown that gonadal H-Ys expression can be manipulated, while non-gonadal H-Ys expression was not affected (reviewed by Zaborski, 1985). These data clearly showed a dual regulation of H-Ys expression in non-mammals: (i) H-Ys is controlled by sex hormones in gonads and thus cannot be the primary determining factor of the gonadal sex, (ii) H-Ys is constitutively expressed in non-gonadal cells according to a non-inducible genetic mechanism.

The H-Ys expression in *Chelonia mydas* (Wellins, 1987) suggests a XX female/XY male/YY male genotypic sex determination. So, it is possible that the male-specific band observed by hybridization of Bkm to genomic DNA reflects the presence of the Y chromosome, although this chromosome is not morphologically differentiated. One published exception is the turtle *Siebenrockiella crassicolis*: the male was shown to present heteromorphic sex chromosomes (Carr & Bickham, 1981), while the female was shown to express high H-Ys level (Engel *et al.* 1981), indicating a possible recessive H-Ys expression in this species.

The typing of H-Ys was used as a tool to reveal genetic sex differences in the European pond turtle (*Emys orbicularis*), a species with TSD and without heteromorphic sex chromosomes. It was studied in different tissues of embryos and juveniles obtained from eggs incubated at 25 °C (100% phenotypic males), 30 °C (100% phenotypic females) and at the pivotal temperature, 28·5 °C (50% males, 50% females). In gonads, the H-Ys expression was shown to depend strictly on phenotypic sex reflecting a control by sex steroid hormones, testes invariably expressing a low H-Ys level (H-Y⁻) and ovaries invariably expressing a high H-Ys level (H-Y⁺) (Zaborski *et al.* 1982). However, the expression of H-Ys antigen in non-gonadal tissues after incubation at extreme temperatures enabled us to define two classes of animals in each sexual phenotype: incubation at 25 °C produced 100% males with either H-Y⁺ or H-Y⁻ phenotype; similarly, incubation at 30 °C produced 100% females with either H-Y⁺ or H-Y⁻ phenotype. On the contrary, there was a large concordance between sexual phenotype and H-Ys level of expression for incubation at pivotal temperature: the males were H-Y⁻ and the females were H-Y⁺ in non-gonadal cells (Zaborski *et al.* 1988). Thus, according to the preceding observations and to the expression of H-Ys in non-mammalian vertebrates, Zaborski *et al.* (1982, 1988) postulated that, in *Emys orbicularis*, the effects of temperature are superimposed upon a genotypic sex determination that correlates with the expression of H-Ys in non-gonadal cells. As there is a striking concordance between H-Ys expression and phenotypic sex for incubation at pivotal temperature, it seems reasonable to assume that there is a linkage between the factor(s) influencing

sex determination at pivotal temperature and the locus of regulation of H-Ys antigen. The expression level of H-Ys is well explained by a one locus/two alleles genetic model in all species studied so far (Wachtel, 1983; Nakamura *et al.* 1987). The expression of H-Ys being high in the ovaries of *Emys orbicularis*, the genetic component in sex determination agrees with a ZZ male/ZW female/WW female genotypic sex determination, demonstrable only within a 1·5 °C window around the pivotal temperature (Zaborski *et al.* 1988). In view of this hypothesis, the H-Ys expression was carried out in blood of adults of *Emys orbicularis* from natural populations. Most females were H-Y$^+$ agreeing with a ZW or WW sexual genotype and the few phenotypic males available at that time for H-Ys typing displayed an H-Ys expression in conformity with a ZZ genotype (Servan *et al.* 1989).

To estimate the importance of this genotypic component in natural populations, we present further data on the Brenne populations of *Emys orbicularis*. Sex ratio and the frequency of sexual inversion in genotypic males and females are estimated. These data, grouped with those of Servan *et al.* (1989), are analysed in two models to estimate the contribution of different genotypes to the sex determination. The first model assumes no influence of both the incubation temperature of eggs and the sexual phenotype on the fitness of individuals. The second hypothesis takes into account an influence of these factors on fitness. In this last model, ESD is favoured over GSD (Charnov & Bull, 1977). Then the consequences on the evolution of ESD are discussed.

2. Material and methods

(i) *Sex ratio definitions*

Etymologically 'Sex ratio' means ratio of the number of individuals of one sex against the other (m/f or f/m with 'f' and 'm' being the numbers of females and males, respectively). Sex ratio is used here as the relative male frequency (m/(m+f)) to simplify the theoretical approach. For genotypic sex determination, primary sex ratio is the sex ratio at fertilization. When temperature-sensitive sex determination occurs, the sexual phenotype is fixed during the thermo-sensitive period of embryonic development. At fertilization, only genotypic predispositions exist. 'Primary Sex Ratio' hereafter refers to sex ratio of all the animals in the population at the embryonic stage when their gonadal sexual phenotype has been irreversibly fixed. According to Lovich & Gibbons (1990), the population sex ratio in turtles is difficult to define. In their study on *Malaclemys terrapin*, they define the 'functional sex ratio' with sexually mature animals although they do not explain exactly what are the criteria of sexual maturity. It seems that secondary sexual characters are exclusively used. However, the secondary sexual characters are not a criterion of

sexual maturity, so there are difficulties in the practical use of this definition. We prefer to consider 'population sex ratio' as the sex ratio of individuals whose sexual phenotype is identified by external morphology.

(ii) *Collection of animals*

All animals came from Brenne, a French region (47° N) where approximately 1200 ponds are interconnected by ditches. Two sampling areas were chosen for the estimation of population sex ratio. Ponds A are a group of seven ponds with free movements of animals from one pond to another (Chopaire, Fontenelle, Neuf, Peaudeux, Pêcherie, Robert, Rosé), and pond B is isolated (Salles; see map in Servan *et al.* 1989). Turtles were captured over nine years with baited traps or hand capture at different times during the activity period and marked individually as previously described (Servan, 1986; Servan *et al.* 1989). Most of the animals were recaptured at least once and the percentage of marked animals in the studied ponds is higher than 95%. Thus, the sex ratio of these animals is a good approximation of the real population sex ratio.

For H-Ys typing, males and females were taken from two areas, ponds A and another group of ponds (ponds C, Etangs Chats) and returned to their respective pond after blood withdrawal. From ponds A, 31 individuals were phenotypic females, 9 were phenotypic males, and 3 were not sexable because of the ambiguity of secondary sexual characters. Among the females, two had been typed 6 years before and thus were used as control of specificity and repeatability of the assay. From ponds C, 14 males and 6 females were caught.

(iii) *Typing of H-Ys antigen*

Blood samples (0·5–3 ml) were withdrawn from the foreleg using a heparinized syringe. Each sample was adjusted to 15 ml with 0·01 M phosphate buffered saline (PBS) and kept at 4 °C for 1 h. Then the supernatant was discarded and the loosely packed cells were resuspended in 15 ml of ice-cold PBS and kept on ice for 1 to 48 h before transfer to the laboratory for H-Ys typing.

The serological H-Y typing of turtle blood cells was performed using an anti-H-Ys antiserum produced in female C57BL/6J Orl mice by repeated intraperitoneal injections of spleen cells from male mice of the same strain (Zaborski *et al.* 1982). The anti-H-Ys specificity was assessed by the absorption of the antiserum with male mice spleen cells resulting in a loss of residual activity of the anti-H-Ys antiserum. Twenty-five μl (turtles from Etangs Chats) or 50 μl (other turtles) of packed blood cells were absorbed with 50μl of anti-H-Ys antiserum (diluted 1/8) for 50 min with gentle shaking every ten min, and centrifuged at 250 *g* for 5 min. The residual anti-H-Ys activity of the super-

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Table 1. *General model for the evolution of alleles 'A' and 'a' involved in the regulation of the H-Ys antigen*

Genotype	AA	Aa	aa
Frequency among newborn offspring	$p_1 p_2$	$p_1 q_2 + p_2 q_1$	$q_1 q_2$
Normalized relative contribution to reproduction among males	$\dfrac{\alpha \sigma_{11}}{Q_1} p_1 p_2$	$\dfrac{\beta \sigma_{21}}{Q_1}(p_1 q_2 + p_2 q_1)$	$\dfrac{\gamma \sigma_{31}}{Q_1} q_1 q_2$
Normalized relative contribution to reproduction among females	$\dfrac{(1-\alpha)\sigma_{12}}{Q_2} p_1 p_2$	$\dfrac{(1-\beta)\sigma_{22}}{Q_2}(p_1 q_2 + p_2 q_1)$	$\dfrac{(1-\gamma)\sigma_{32}}{Q_2} q_1 q_2$
H-Ys status for D hypothesis	H-Y$^-$	H-Y$^+$	H-Y$^+$
H-Ys status for R hypothesis	H-Y$^-$	H-Y$^-$	H-Y$^+$

$$Q_1 = \alpha \sigma_{11} p_1 p_2 + \beta \sigma_{21}(p_1 q_2 + p_2 q_1) + \gamma \sigma_{31} q_1 q_2$$
$$Q_2 = (1-\alpha)\sigma_{12} p_1 p_2 + (1-\beta)\sigma_{22}(p_1 q_2 + p_2 q_1) + (1-\gamma)\sigma_{32} q_1 q_2$$
$$H = \alpha p_1 p_2 + \beta(p_1 q_2 + p_2 q_1) + \gamma q_1 q_2$$

p_1 is the frequency of 'A' allele in males and $q_1 = 1 - p_1$ is the frequency of 'a' allele in males. p_2 is the frequency of 'A' allele in females and $q_2 = 1 - p_2$ is the frequency of 'a' allele in females. The frequency of males is α for individuals of genotype AA, β for individuals of genotype Aa and γ for individuals of genotype aa. Moreover, each of them has a different fitness, σ_{ij} with i defining the genotype (1 for AA, 2 for Aa and 3 for aa) and j equal 1 for males or 2 for females. H is the primary sex ratio and Q_1 and Q_2 are normalizing factors.

natant was then evaluated according to the technique of Zaborski (Zaborski, 1979; Zaborski *et al.* 1982) modified as follows.

One volume (25 μl) of the absorbed supernatant was mixed with one volume of a suspension of mouse spermatozoa for 30 min at 37 °C.

Spermatozoa were washed twice by centrifugation in gelatin buffer (0·3 % gelatin in 0·01 M PBS).

The sperm pellet was adjusted to one volume in PBS, and one volume of rabbit anti-mouse IgG (Cappel Laboratories), diluted 1/40, was added for 30 min at 37 °C.

After 3 washes in gelatin buffer, the sperm pellet was resuspended in PBS to one volume and mixed with one volume of protein A, β-galactosidase linked (Amersham), diluted 1/100, for 3 h at 37 °C.

Then, after 2 washes in gelatin buffer, the sperm pellet was resuspended in 100 μl of the β-galactosidase substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG, 3 mM, Amersham) for 1 h at 37 °C.

The reaction was stopped by adding 100 μl of 1 M Na_2CO_3 and the colour intensity was measured by the absorbance (O.D., Optical Density) at 410 nm with a spectrophotometer.

A low O.D. value denotes a low residual anti-H-Ys activity after absorption with turtle cells displaying a high level of H-Ys expression. These cells therefore were identified as H-Ys positive (H-Y$^+$), whereas a high O.D. value revealed H-Ys negative cells (H-Y$^-$).

As blood cell samples were processed in four series, each of them using spermatozoa from four different male mice, results are presented separately for each series. Animals of series 1–3 are from ponds A and animals of series 4 are from ponds C.

(iv) *Genetic contribution to sex determination*

We assume that in *Emys orbicularis* H-Ys antigen is regulated by a one gene/two alleles genetic system (see Introduction). Let 'A' and 'a' be these two alleles. The 3 types of individual can be H-Y$^+$ or H-Y$^-$ in respect to their genotype AA, Aa or aa. As there is no direct evidence that H-Y$^+$ is dominant over H-Y$^-$ in *Emys orbicularis*, we will test here both hypotheses (called hypothesis D for H-Y$^+$ dominant and hypothesis R for H-Y$^+$ recessive). In the hypothesis of H-Y$^+$ dominant, individuals H-Y$^-$ are of genotype AA, and individuals H-Y$^+$ are of genotype Aa and aa. In the hypothesis of H-Y$^+$ recessive, individuals H-Y$^-$ are of genotype AA and Aa and individuals H-Y$^+$ are of genotype aa. In both hypotheses, a genotypic AA animal is H-Y$^-$ and a genotypic aa animal is H-Y$^+$.

Two models are analysed. In the first one, differential fitness as a function of sexual phenotype and temperature incubation of eggs are not taken into account. In this model we assume non-overlapping generations, random sampling of gametes and mendelian segregation of characters. For the second model, each individual has a fitness depending on its sexual phenotype and on incubation temperature during its embryonic life. According to Charnov & Bull (1977), this condition favours ESD over GSD. The fitness is expected to act by differential fertility due to change in growth as a function of the incubation temperature but not on survivorship. Then, we can estimate that the frequencies of H-Y$^+$ and H-Y$^-$ animals among one sex are the same for the hatchlings and the animals of the population.

Individual fitness parameters will be used because females can mate several times during one season and can store the sperm alive for more than one year. The general form of the transformation equations is shown in Table 1.

The values are presented as mean $\pm$ standard deviation and all the tests are performed at the confidence limit of 5 %. Unknown covariances are neglected to estimate standard deviation of primary sex ratio (H) and frequency of H-Y$^+$ individuals among adults (n_p).

The parameters used in this study are defined in the Appendix.

3. Results

(i) *Population sex ratio*

The sex ratio of sexable marked turtles (*Emys orbicularis*) captured in ponds A was 0·33 (151 males and 306 females) that is significantly different from 0·5 ($\chi^2 = 52·57$, D.F. = 1, $P < 0·0001$). The sex ratio in pond B was 0·38 (14 males and 22 females) that is not significantly different from 0·5 ($\chi^2 = 1·77$, D.F. = 1, $P > 0·15$). Two other ponds were previously studied, Ricot (15 males and 35 females) and Pied du Tour (16 males and 27 females) (Servan *et al.* 1989). Taking into account these previous results, no significant differences between the ponds appeared by comparing them together ($\chi^2 = 1·11$, D.F. = 3, $P > 0·35$). In all ponds studied, the sex ratio of marked animals was $0·33 \pm 0·04$ and it will be considered as the sex ratio of populations in Brenne.

(ii) *H-Ys typing*

Data are shown in Fig. 1. Twenty out of the 23 males are H-Y⁻ and 35 out of the 37 females are H-Y⁺. By grouping the present data with those published by Servan *et al.* (1989), a total of 113 females and 27 males were H-Y-typed. Among the females, 106 are H-Y⁺ and 7 are H-Y⁻, corresponding to a frequency
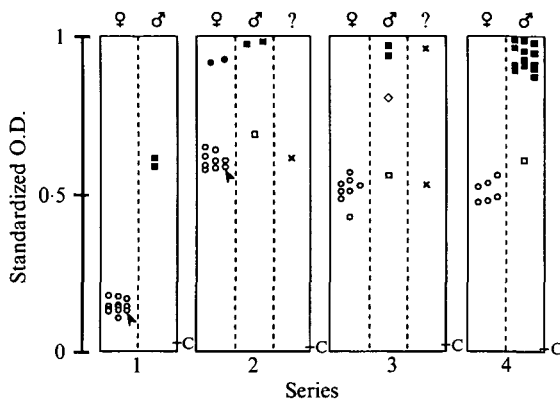

Fig. 1. Residual activity of mouse H-Ys antiserum after absorption with blood cells from adult males and females of *Emys orbicularis*. Each point represents one determination in duplicate done on one individual. The sample values of OD at 410 nm were standardized to the following actual values obtained with control unabsorbed H-Ys antiserum (taken as 1): $OD_{series 1} = 0·49$; $OD_{series 2} = 1·37$; $OD_{series 3} = 1·65$; $OD_{series 4} = 1·26$. −C, represents control background OD in suspensions containing spermatozoa + rabbit anti-mouse IgG + protein A, β-galactosidase linked, but no H-Ys antiserum. The arrowheads indicate the two females invariably H-Y⁺ over a 6-year interval. ■ = Phenotypic male, H-Ys negative; □ = Phenotypic male, H-Ys positive; ● = Phenotypic female, H-Ys negative; ○ = Phenotypic female, H-Ys positive; ◇ = Phenotypic male, intermediate H-Ys level; × = Sexual phenotype ambiguous.
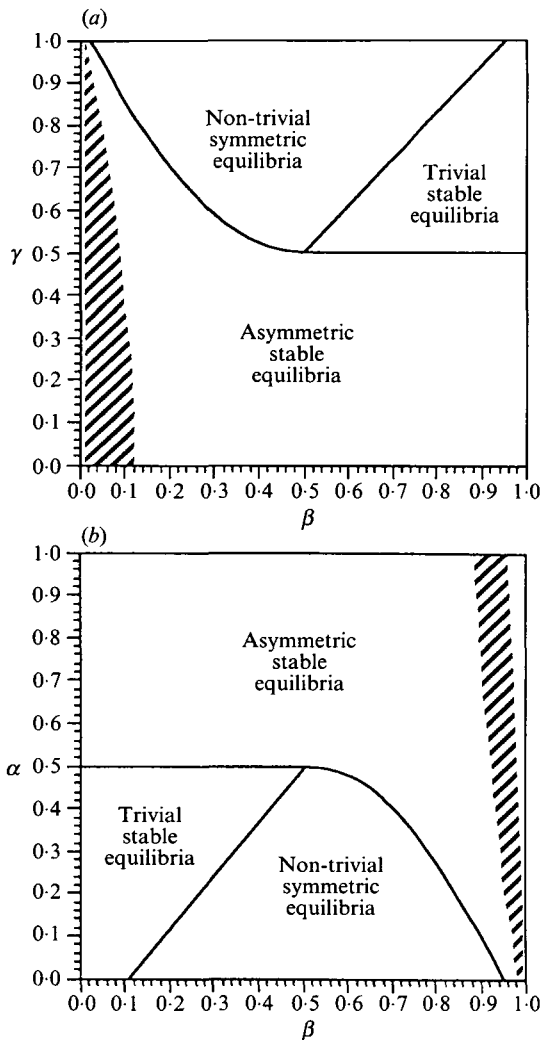
of H-Y⁺ females $n_f = 0·94 \pm 0·02$. Among the males, 3 are H-Y⁺ and 24 are H-Y⁻, corresponding to a frequency of H-Y⁺ males $n_m = 0·11 \pm 0·06$. The two females typed twice over a 6-year interval invariably exhibited the same H-Y⁺ phenotype. The sex ratio of animals typed for H-Ys is more biased than the population sex ratio because many of them were females caught specially to obtain eggs. According to the data of Zaborski *et al.* (1982, 1988), the frequency of H-Y⁺ animals among 159 hatchlings and young raised in the laboratory at 25, 28·5 and 30 °C was $n_H = 0·54 \pm 0·04$. It is important to note that the H-Ys phenotype used here is the non-gonadal H-Ys phenotype which is insensitive to the incubation temperature (Zaborski *et al.* 1988).

(iii) *Primary sex ratio*

The frequency of H-Y⁺ animals in phenotypic females (n_f) and in phenotypic males (n_m) is expected to be the same in hatchlings and in adults if no difference in fitness occurs or if some fitness acts in one or both sexes by differential fertility. The primary sex ratio H in the population can be estimated using $n_H = n_f \times (1 - H) + n_m \times H$.

$$H = (n_H - n_f)/(n_m - n_f) = 0·49 \pm 0·06.$$

(iv) *Primary sex ratio* vs. *Population sex ratio*

We have shown that the population sex ratio is female-biased: $P = 0·33 \pm 0·04$. Servan *et al.* (1989) have proposed that this bias could be explained by a bias of primary sex ratio. We can reject this hypothesis because the primary sex ratio in Brenne estimated above is $H = 0·49 \pm 0·06$. The differential migration and bias in capture have been previously excluded to explain the bias of population sex ratio. The age at maturity is higher in females than in males and therefore this parameter cannot explain a female-bias of population sex ratio. The last solution is a differential survival of females *vs.* males. This is consistent with the finding that the main factor influencing population sex ratio for long life-span species is the differential survival between sexes (Girondot & Pieau, 1993). With higher survival in phenotypic females, we can expect that the frequency of H-Y⁺ animals is higher in adults (n_P) than in hatchlings (n_H) because females are mainly H-Y⁺ animals. It is really what we observe: $n_P = (1 - P) n_f + P n_m = 0·67 \pm 0·04$, $n_H = 0·54 \pm 0·04$, $\epsilon = 2·29$, $P < 0·02$. This differential survival between males and females does not invalidate the hypothesis that fitness does not act by mortality. Indeed, here, the death of a male or a female is independent of its H-Ys phenotype.

(v) *Genetic contribution to sex determination*

The values of the parameters n_f, n_m, n_H and H have been evaluated from natural populations and will be

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Fig. 2. Stable equilibria if H-Y$^+$ is dominant for $\alpha = 0.956$ (hypothesis D, Fig. 2a) and if H-Y$^+$ is recessive for $\gamma = 0.103$ (hypothesis R, Fig. 2b). The hatched zone of asymmetric stable equilibria (primary sex ratio equals 0.5) defines where n_f, n_m and n_H are compatible with values observed in hatchlings and in population.

used to estimate the probability for each genotype to differentiate as male or female in a one locus/two alleles genetical model of regulation of H–Ys.

(a) *Model 1*. In this model we assume that both temperature and sexual phenotype have no influence on fitness. If fitness is influenced only by one of these factors, this model can be applied. Among the theoretical models on sex ratio evolution including genetic and epigenetic components for sex determination, Scudo (1964) proposed a model in which two alleles of one locus were implicated. This model was in part solved by Eshel (1975) and the resolution was completely achieved by Karlin & Lessard (1986). As the fitness values are equal, $\sigma_{11} = \sigma_{12} = \sigma_{21} = \sigma_{22} = \sigma_{31} = \sigma_{32} = 1$ (see Table 1 for the complete model). Let α, β and γ be the frequencies of males within the determined systems AA, Aa or aa respectively. There are 3 categories of stable equilibrium: (i) one of the

two alleles is fixed (called trivial solution); (ii) both alleles are at the same frequency in males and females and bias of sex ratio occurs (called symmetric solution) and (iii) the frequency of both alleles is different in males and females, and the sex ratio is 0.5 (called asymmetric solution).

In the hypothesis D (H-Y$^+$ dominant), where the H-Ys expression in Aa animals is not distinguished from that in aa animals (both are H-Y$^+$), we can evaluate the α value but cannot distinguish β and γ. We designate by (AA), (Aa) and (aa) the relative frequency of AA, Aa and aa individuals and by suffixes [f] and [m] this frequency in phenotypic females and phenotypic males respectively. If H is the primary sex ratio, the frequency of phenotypic males of genotype AA $(AA[m])$ is $H(1-n_m)$, and the frequency of phenotypic females with genotype AA $(AA[f])$ is $(1-H)(1-n_f)$, so the α value is:

$$\alpha = [H(1-n_m)]/[H(1-n_m)+(1-H)(1-n_f)] = 0.956.$$

All the equilibria have been investigated for $(\alpha = 0.956)$ and $(0 < (\beta, \gamma) < 1$ step $0.01)$. Non-trivial stable equilibria are achieved for the symmetric solution with $(0.50 < H < 1.00)$ and for the asymmetric solution with $(H = 0.5)$. For each stable equilibrium, the theoretical $\hat{n}_f$, $\hat{n}_m$ and $\hat{n}_H$ values have been computed. The stable equilibria with $n_f - 2 \times SD < \hat{n}_f < n_f + 2 \times SD$, $n_m - 2 \times SD < \hat{n}_m < n_m + 2 \times SD$ and $n_H - 2 \times SD < \hat{n}_H < n_H + 2 \times SD$ are retained. Only symmetric stable equilibria $(H = 0.5)$ show genetical structure compatible with the values obtained from population. In these conditions, there is a high constraint on the β value $(0.01 \leqslant \beta \leqslant 0.12)$, but not on the γ value (Fig. 2a). The frequency of aa individuals $(0.002 \leqslant (aa) \leqslant 0.211)$ is always lower than the frequencies of Aa $(0.479 \leqslant (Aa) \leqslant 0.571)$ and AA $(0.282 \leqslant (AA) \leqslant 0.517)$ individuals.

In the hypothesis R (H-Y$^+$ recessive), where the H-Ys expression in Aa animals is not distinguished from that in AA animals (both are H-Y$^-$), we can determine the γ value but cannot distinguish α and β. The frequency of phenotypic males of genotype aa $(aa[m])$ is Hn_m, and the frequency of phenotypic females with genotype aa $(aa[f])$ is $(1-H)n_f$, so the γ value is:

$$\gamma = [Hn_m]/[Hn_m+(1-H)\,n_f] = 0.103.$$

All the equilibria have been studied for $(\gamma = 0.103)$ and $(0 < (\alpha, \beta) < 1$ step $0.01)$. Non-trivial stable equilibria are achieved for the symmetric solution with $(0.00 < H < 0.50)$ and for the asymmetric solution with $(H = 0.5)$. For each stable equilibrium, the theoretical values $\hat{n}_f$, $\hat{n}_m$ and $\hat{n}_H$ have been computed. The stable equilibria with $n_f - 2 \times SD < \hat{n}_f < n_f + 2 \times SD$, $n_m - 2 \times SD < \hat{n}_m < n_m + 2 \times SD$ and $n_H - 2 \times SD < \hat{n}_H < (n_H + 2)SD$ are retained. Only symmetric stable equilibria $(H = 0.5)$ show genetical structure compatible with the values obtained from the population. A high constraint on the β value is

observed ($0.88 \leqslant \beta \leqslant 0.99$), but not on the α value (Fig. 2*b*). For these solutions, the frequency of *AA* individuals ($0.004 \leqslant (AA) \leqslant 0.073$) is always lower than the frequencies of *Aa* ($0.447 \leqslant (Aa) \leqslant 0.498$) and *aa* ($0.457 \leqslant (aa) \leqslant 0.546$) individuals.

In this model, there is a genetic variation in sex ratio and a second phenotype (H-Ys) is mapped onto the genetic modifiers of sex ratio. Since all genotypes within a sex have equal fitness, the genetic modifiers are selectively neutral traits. The evolutionary stable primary sex ratio will necessarily be 0.5 (Fisher, 1929) which is compatible with the value estimated for the natural population, $H = 0.49 \pm 0.06$, and the H value obtained in the model, $H = 0.5$.

The generalization of the results for both hypotheses D and R can be made as follows.

One of the homozygotes is mainly of one sex, and the heterozygote is mainly of the other one.

There is low constraint on sex determination of the second homozygote which is at the lower frequency in the population.

(*b*) *Model 2*. In the second model, each individual has a fitness influenced by its sexual phenotype and temperature of incubation. A differential effect of environment according to sexual phenotype on fitness was proposed by Charnov & Bull (1977) to explain the advantage of ESD over GSD. We consider here that fitness modifies the fertility of individuals, but not their survival. Such a model was previously described (Bull, 1981; Karlin & Lessard, 1984) but the general form of the equilibria was missing because of the higher number of parameters to consider. As the distribution of fitness for both sexes as a function of temperature is not known, we need only 6 parameters to estimate the frequencies of alleles at equilibrium. Without loss of generality, let $\alpha_1 = \sigma_{11}\alpha$ and $\alpha_2 = \sigma_{12}(1-\alpha)$, $\beta_1 = \sigma_{21}\beta$ and $\beta_2 = \sigma_{22}(1-\beta)$, $\gamma_1 = \sigma_{31}\gamma$ and $\gamma_2 = \sigma_{32}(1-\gamma)$ be the effective contribution to reproduction of the different genotypes as male or female with

$$0 \leqslant \alpha_1 \leqslant 1, 0 \leqslant \alpha_2 \leqslant 1, (\alpha_1 + \alpha_2) \leqslant 1$$

$$0 \leqslant \beta_1 \leqslant 1, 0 \leqslant \beta_2 \leqslant 1, (\beta_1 + \beta_2) \leqslant 1$$

$$0 \leqslant \gamma_1 \leqslant 1, 0 \leqslant \gamma_2 \leqslant 1, (\gamma_1 + \gamma_2) \leqslant 1$$

All stable equilibria have been studied by computer analysis for all the $(\alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_2)$ possible values by steps of 0.1. For each value, the convergence is analysed with initial frequency of p_1 (frequency of allele *A* among males) and p_2 (frequency of allele *A* among females) from 0 to 1 (step 0.1). The validation of global convergence derives from the monotonicity properties of the transformation equations of frequencies from one generation to another (Karlin & Lessard, 1986). The iterations have been stopped when the p_1 and p_2 frequencies are stabilized at $\pm 10^{-5}$. It can be easily demonstrated that the fixation of an allele is a stable equilibrium (called trivial symmetric
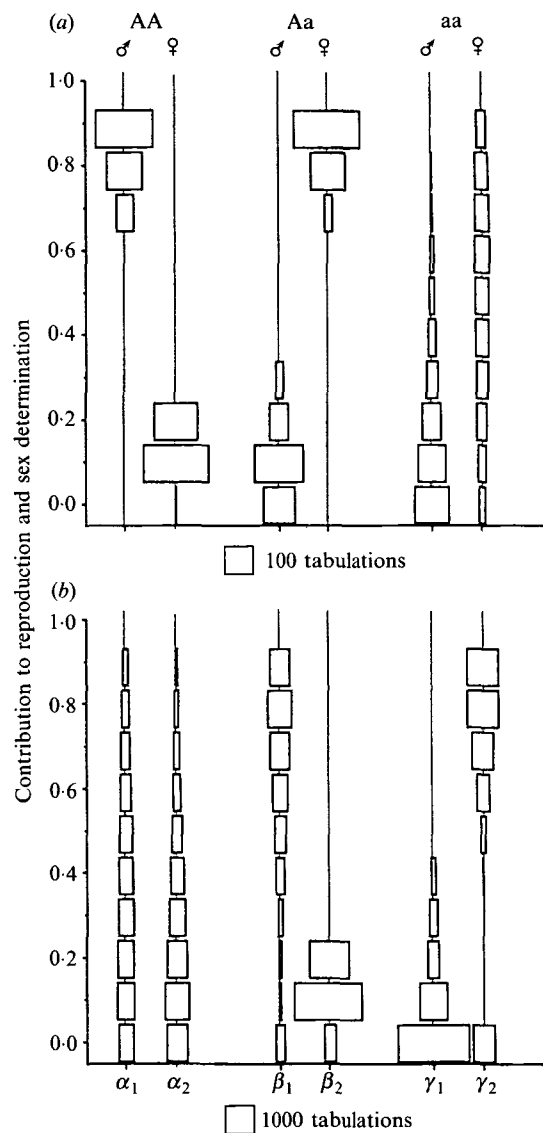

Fig. 3. Contribution to reproduction as male or female of the individuals according to their genotype for D hypothesis (*a*, H-Y$^+$ dominant) or R hypothesis (*b*, H-Y$^+$ recessive). In this model the fitness acts by influencing the fertility of individuals. The width of each rectangle is proportional to the number of tabulations with the corresponding value of contribution to reproduction.

stable equilibrium). For each stable equilibrium, another iteration has been performed for all the α, β and γ compatible values ($\alpha_1 \leqslant \alpha \leqslant 1 - \alpha_2, \beta_1 \leqslant \beta \leqslant 1 - \beta_2, \gamma_1 \leqslant \gamma \leqslant 1 - \gamma_2$ step 0.05). As for the model 1, only solutions with the theoretical values n_f, n_m, H and n_H compatible with the population values ($\pm 20 \times$ SD) are retained. The equations used are defined in Table 1.

For D hypothesis, 277 combinations of the ($\alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_1$) parameters over the 23×10^6 combinations possible are compatible with the data estimated for the Brenne population. The distribution of the ($\alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_2$) values for these stable equilibria is shown in Fig. 3*a*. We show clearly that *AA* animals reproduce mainly as males ($\alpha_1 > \alpha_2$) and *Aa* animals

reproduce mainly as females ($\beta_1 < \beta_2$). There is no high constraint for the reproduction of *aa* animals (Fig. 3*a*). The frequency of *AA* individuals is within the range [0·38, 0·50], the frequency of *Aa* individuals within the range [0·47, 0·56] and the frequency of *aa* individuals within the range [0·02, 0·12].

For R hypothesis, a total of 3585 combinations of ($\alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_2$) parameters are compatible with the data estimated for the Brenne population. The distribution of the ($\alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_2$) values for these stable equilibria is shown in Fig. 3*b*. We show that *Aa* animals reproduce mainly as males ($\beta_1 > \beta_2$) and *aa* animals reproduce mainly as females ($\gamma_1 < \gamma_2$). There is no high constraint for the reproduction of *AA* animals (Fig. 3*b*). However, these conclusions are not as strict as that for the D hypothesis. For example, in 630 cases, *aa* individuals do not reproduce as females ($\gamma_2 = 0$) but can reproduce as males ($\gamma_1 \geqslant 0$). The frequency of *AA* individuals is within the range [0·01, 0·10], the frequency of *Aa* individuals within the range [0·34, 0·50] and the frequency of *aa* individuals within the range [0·46, 0·62].

We can expect the fitness values (σ_{ij}) to be not very different from unity since up to now the tentatives to measure some differences of fitness as a function of temperature in reptiles with TSD have failed. Then we conclude that α, β and γ are not very different from α_1, β_1 and γ_1 respectively, and $1 - \alpha$, $1 - \beta$ and $1 - \gamma$ are not very different from α_2, β_2 and γ_2 respectively.

(*c*) *Generalization of the results for both models.* In both models, we observe that one kind of homozygous animals differentiates mainly as one sex and the heterozygous animals differentiate mainly as the other sex. The other homozygote can differentiate either as a male or a female without high constraint. These last homozygous animals are always at the lower frequency in the population. These conclusions are not so strict in model 2 when H-Y$^+$ is recessive, but this hypothesis is the less probable because H-Y$^+$ is dominant in the great majority of species (Wachtel, 1983; Nakamura *et al.* 1987).

4. Discussion

In this study, the sex determination in a turtle (*Emys orbicularis*) is considered to be influenced by both temperature and a genetic component. The genetic component is reflected by the expression of the serologically defined H-Y antigen in non-gonadal cells. This expression was used as a marker of genotypic sex, genotypic females being H-Y$^+$ and genotypic males being H-Y$^-$.

We show that in the majority of individuals of adult populations in Brenne, the sexual phenotype and the expression of H-Ys antigen are concordant [$(P - (n_m P)) + (1 - P) - ((1 - n_f)(1 - P)) = 0.91$]. The other individuals (6% of the females and 11% of the males) are sexually inverted. In these individuals, the conditions of incubation temperature of eggs

were either feminizing [$(1 - n_f) \times 100 = 6\%$] or masculinizing [$n_m \times 100 = 11\%$] and overrode the influence of the genetic component. To estimate the percentage of animals incubated in conditions allowing the genetic component to influence sex determination, the genotypic males (H-Y$^-$) incubated at masculinizing temperature ($n_m H$) and the genotypic females (H-Y$^+$) incubated at feminizing temperature ($(1 - n_f)(1 - H)$) must be subtracted from the number of animals in which H-Ys phenotype and sexual phenotype are concordant. Then the proportion of eggs incubated in conditions of temperature allowing the genotypic component to influence sex determination is:

$$H - ((1 - n_f) H + n_m H) + (1 - H) - ((1 - n_f)$$
$$(1 - H) + n_m(1 - H)) = n_f - n_m = 0.83,$$

assuming that the sex-determining factor is in maximal linkage disequilibrium with H-Ys as shown by Zaborski *et al.* (1988). These conclusions are independent of the nature of the regulation of H-Ys, by one locus/two alleles or multigenic.

Assuming that the H-Ys antigen is regulated by two alleles *A* and *a*, we show that one category of homozygous animals (*AA* or *aa*) reproduces mainly as one sex and the heterozygous animals (*Aa*) reproduce mainly as the other sex. The other category of homozygous animals is at low frequency in the population and reproduces as male or female without high constraint. Thus the genetic component of sex determination in *Emys orbicularis* accounts for a system of monogenic genotypic sex determination. This turtle is perhaps on the way to acquiring strict ESD, if ESD is apomorphic, or to acquiring strict GSD, if GSD is apomorphic. The discussion of the origin of sex determination in reptiles remains open. A plesiomorphic environmental sex determination appears to be more parsimonious than genotypic sex determination for reptiles (Janzen & Paukstis, 1991*a*), but ESD can be adaptive and therefore apomorphic (Bull, 1983). The linkage between sex determining genes and the locus of regulation of H-Ys could be selected because H-Ys seems to be involved in gametogenesis at least in mammals. Logically this linkage would be ancestral in amniotes and ESD would be an apomorphic character. *Emys orbicularis* has both ESD and a linkage between sex determining genes and the locus of regulation of H-Ys. As approximately 91% of animals have phenotypic sex corresponding to their genotypic sex, this linkage can still be selected.

As already quoted, there are some laboratory studies showing a genotypic contribution to sex determination in reptiles with ESD. *Emys orbicularis* is another species with both ESD and GSD. One way to demonstrate the action of a genetic factor on one character is to estimate the heritability of this character. At pivotal temperature, the heritability of the zygotic character of sex ratio in the turtle

Graptemys ouachitensis is 0·82 (Bull *et al.* 1982). This heritability is 'rather high for a quantitative genetic character' and a major gene could be implicated in the sex determination at pivotal temperature. The same heritability was obtained in *Chelydra serpentina*, and this high heritability was specified as not due to genotypic-environment interactions (Janzen, 1992). However, most natural nests of *Graptemys ouachitensis* in one locality are unisexual (Vogt & Bull, 1984). So, in nature the genetic component at the pivotal temperature has little influence on sex determination in that locality. By contrast, 115 embryos of *Emys orbicularis* from 27 clutches were field-developed in five experimental series. In each of them, males and females were obtained simultaneously (Pieau, 1982). We can interpret these results by biological differences but also by ecological differences between species. Particularly it would be very important to know the temperature in natural nests during the thermosensitive period of development.

Bull *et al.* (1982) postulated that the effective heritability (or heritability of the zygotic character of sex ratio in natural conditions) is much lower than the heritability at pivotal temperature because most of the nests are incubated at extreme temperatures (all masculinizing temperature or all feminizing temperature). Our data for *Emys orbicularis* do not agree with this hypothesis. The fluctuations of temperature could be an important factor. Most experiments in laboratories are conducted at constant temperature (but see Pieau, 1973; Bull & Vogt, 1979; Wilhoft *et al.* 1983; Paukstis *et al.* 1984), whereas in natural conditions temperature fluctuates. When the temperature fluctuates around the pivotal temperature during the thermosensitive period, the effective heritability would not be much reduced in comparison with an incubation at constant pivotal temperature.

Appendix

Parameters evaluated from natural populations

n_f Frequency of H-Y$^+$ individuals among phenotypic females

n_m Frequency of H-Y$^+$ individuals among phenotypic males

H Primary sex ratio (frequency of males in hatchlings)

n_H Frequency of H-Y$^+$ individuals among hatchlings

P Population sex ratio (frequency of males among adults)

n_P Frequency of H-Y$^+$ individuals among adults

Parameters used for modelization

p_1 Frequency of A allele in males

p_2 Frequency of a allele in males ($p_2 = 1 - p_1$)

q_1 Frequency of A allele in females

q_2 Frequency of a allele in females ($q_2 = 1 - p_2$)

α Frequency of males among individuals of genotype AA

β Frequency of males among individuals of genotype Aa

γ Frequency of males among individuals of genotype aa

σ_{11} Mean relative fitness in males of genotype AA

σ_{12} Mean relative fitness in females of genotype AA

σ_{21} Mean relative fitness in males of genotype Aa

σ_{22} Mean relative fitness in females of genotype Aa

σ_{31} Mean relative fitness in males of genotype aa

σ_{32} Mean relative fitness in females of genotype aa

α_1 Relative contribution to reproduction of individuals AA as males

α_2 Relative contribution to reproduction of individuals AA as females

β_1 Relative contribution to reproduction of individuals Aa as males

β_2 Relative contribution to reproduction of indivdiuals Aa as females

γ_1 Relative contribution to reproduction of individuals aa as males

γ_2 Relative contribution to reproduction of individuals aa as females

Q_1, Q_2 Normalizing factors

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