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# Feminizing Turtle Embryos as a Conservation Tool

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**Abstract:** *Most turtles have temperature-dependent sex determination. With the intention to preserve endangered and threatened species, two management tools have been suggested: artificial incubation at either male- or female-producing temperatures and introduction of many more females than males into populations by manipulating incubation temperatures to favor the production of female embryos. The use of estrogens during incubation has also been proposed to induce the development of females. We argue that in nature the incubation of eggs around the pivotal temperature is probably more frequent than generally recognized and leads to either phenotypic adult females or phenotypic adult males, even though the embryonic testis may present various degrees of intersexuality, including ovotestes as documented in artificial incubation. Observations of the turtle *Emys orbicularis* show that, after hatching, ovotestes tend to evolve into testes by regression, total or partial, of the ovarian-like cortex. Testes with some immature oocytes at their surface have been observed in adult turtles, and they produce spermatozoa. Therefore, gonadal intersexuality apparently does not hinder the reproductive male function in adults. We draw attention to the danger of estrogenic treatment of embryos to produce females. In many cases, such treatment induces thin gonads (“hypogonads”) in which the volume of both cortex and medulla are reduced. Exogenous estrogens may also result in the arrest of lengthening of the Müllerian ducts and sometimes in the opening of their caudal end in the Wolffian ducts. Either process results in the inability of adult females to evacuate eggs from oviducts. We modeled the long-term effects of introducing in a population a strongly female-biased primary sex ratio (20 females to 1 male) for 30 years. Taking into account a genetic component of sex determination, as exemplified by the results of incubation at pivotal temperature, such a manipulation favors masculinizing alleles. When the manipulation is stopped, the primary sex ratio, as well as the adult sex ratio, becomes male-biased—a result contrary to that expected—and the population size decreases. We recommend actions that protect adult populations and nesting sites and that improve the natural conditions of incubation in these sites rather than attempts to manipulate sex ratios.*

Feminización de Embriones de Tortugas Como una Herramienta de Conservación

**Resumen:** *La mayoría de las tortugas poseen determinación sexual dependiente de la temperatura. Con la intención de preservar especies en peligro o amenazadas, dos herramientas de manejo han sido sugeridas: Incubación artificial a temperaturas tanto productoras de machos como de hembras y la introducción de mucho más hembras que machos en las poblaciones mediante la manipulación de las temperaturas de incubación para favorecer la producción de embriones de hembras. El uso de estrógenos durante la incubación ha sido propuesto para inducir el desarrollo de hembras. Nosotros argumentamos que en la naturaleza, la incubación de huevos alrededor de temperaturas pivotes es probablemente más frecuente de lo que en general se ha reconocido, tendiendo a producir organismos adultos fenotípicamente hembras o fenotípicamente machos, aunque los testículos podrían presentar diversos grados de intersexualidad, incluyendo ovotestis, como se ha documentado en incubaciones artificiales. Observaciones en la tortuga *Emys orbicularis* muestran que después de la eclosión los ovotestis tienden a evolucionar hacia testículos por regresión total o parcial de la corteza de tipo ovárica. En algunos organismos adultos se han observado testículos con algunos ovocitos inmaduros en la superficie, sin embargo estos producen espermatozoides. Por lo tanto, la intersexualidad go-*

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*nadal aparentemente no inhibe la función reproductiva del macho en adultos. Nosotros pusimos atención en el riesgo de producir embriones de hembras mediante tratamietos estrogénicos. En muchos casos, estos tratamientos indujeron gonadas pequeñas (Hipogónadas) en las cuales el volúmen tanto de la corteza como de la médula son muy reducidos. Estrógenos exógenos pueden también resultar en el arresto del elongamiento de los conductos Müllerianos y algunas veces de su apertura final en los conductos de Wolff, cualquiera de estos procesos imposibilita a los organismos adultos para la evacuación de huevos de los oviductos. Modelamos los efectos a largo plazo de la introducción en una población de sexos fuertemente sesgados hacia hembras (20 hembras; 1 macho) por 30 años. Tomando en consideración un componente genético de la determinación sexual, como se ha ejemplificado en los resultados de incubaciones a temperaturas pivote, favoreciendo los alelos masculinizantes. Cuando la manipulación es detenida, la proporción sexual primaria, así como la proporción sexual de los adultos se torna sesgada hacia machos. Un resultado contrario a lo esperado, disminuyendo el tamaño poblacional. Recomendamos acciones para proteger las poblaciones de adultos y los sitios de anidamiento para mejorar las condiciones de incubación natural, en lugar de llevar a cabo intentos de manipulación de las proporciones de sexos.*

## Introduction

The demography of turtles is characterized by both a relatively low probability of survival for embryos and hatchlings and a relatively high probability of annual survival for adults (Iverson 1991). The low probability of embryonic survival may have four causes: predation of eggs by humans or other animals, destruction of embryos by abiotic factors (e.g., thermal extremes, humidity extremes), microbial infection, and destruction of nests by other nesting females. Thus, egg and embryo survival can be enhanced by protecting nesting sites, transplanting the eggs to protected sites, or incubating eggs under controlled conditions. But the process of sex determination can be altered by any of these three actions. The sexual phenotype is influenced by incubation temperature during a critical period of embryonic development for most turtle species (temperature-dependent sex determination, TSD; Crews et al. 1994; Pieau et al. 1994a). If the eggs are transplanted, the incubation temperature can be modified; this is a serious problem in hatcheries. Several years ago the danger of masculinizing turtle embryos by incubating eggs in artificial conditions was pointed out, and attention was drawn to the need for monitoring temperature in hatcheries to ensure that both sexes are produced (Mrosovsky & Yntema 1980; Mrosovsky 1982).

As in many other reptiles, the population growth in turtles is generally female-limited because the number of offspring is directly related to the number of reproductive females in the population (Girondot & Pieau 1996). Thus, the use of artificial incubation for conservation of species with TSD has been proposed with the following recommendations: (1) incubate eggs at either feminizing or masculinizing temperatures to control precisely the process of sex determination, (2) do not incubate eggs at or near the pivotal temperature (temperature producing both sexes in equal proportion) to avoid the produc-

tion of intersexes, and (3) produce far more females than males with a ratio of 1 male for 6 to 20 females (Vogt 1994).

Mrosovsky and Godfrey (1995) and Lovich (1996) have already commented on these recommendations. Mrosovsky and Godfrey reviewed the data dealing with the sex ratios in natural nests of marine turtles (Cheloniidae and Dermochelyidae) and showed that both sexes are frequently found together in many natural nests. They argued that the high percentage of intersexes reported previously among hatchlings of *Dermochelys coriacea* (Benabib Nisenbaum 1984) was an artifact of the fixation method used. Finally, they drew attention to the need for more information before sex ratios of turtle embryos are manipulated. Likewise, Lovich (1996) considered that the available information argues against sex-ratio manipulation because it can change the process of sexual selection and destabilize the resources used by both sexes. We contribute to the debate by examining three points: the phenomenon of intersexuality in turtle embryos and its evolution during life; different feminizing methods and their consequences and risks to the individuals involved; and the long-term consequence of feminizing turtle populations.

## The Meaning and Evolution of Intersexuality

It has been claimed that in TSD sex determination is an all-or-none process and that intersexes are rarely formed (Crews et al. 1994). Nevertheless, several cases of intersexuality have been reported in hatchling, young, and adult turtles known to have TSD. Intersexuality concerns the morphological condition of gonads (ovotestes) and/or genital ducts (persistence of Müllerian ducts in males and rudiments of epididymis and vas deferens in females) (reviewed by Forbes 1964; Raynaud & Pieau 1985). In the European pond turtle (*Emys orbicularis*),

gonadal intersexuality has been observed in late embryonic stages as well as at hatching, in both artificial and natural conditions of egg incubation (Pieau 1974a, 1976, 1982; Pieau & Dorizzi 1981). Thus, at 28.5°C (pivotal temperature), testes and ovaries can be found, but a cortex with various degrees of development is often present at the surface of testes. In many cases these testes exhibit an ovary-like cortex with numerous germ cells in meiotic prophase; they have been defined as ovotestes (Pieau 1976; Raynaud & Pieau 1985). In natural nests the incubation temperature is not constant but fluctuates with night and day and with weather changes. When temperature fluctuations are above and below the pivotal temperature during the thermosensitive period, intersexes are often produced just as in artificial incubation (Pieau 1982).

We have examined the structure of ovotestes in *Emys orbicularis* individuals hatched from eggs incubated at 28.5°C. This examination was carried out at hatching and at 1, 4, and 9 months after hatching. We have observed that at hatching oocytes begin to degenerate into the cortex. Degeneration continues after hatching, so ovotestes generally evolve into typical testes. But some oocytes can escape degeneration, and at 9 months some growing oocytes are sometimes observed at the surface of testes (Girondot 1993). In a previous paper we hypothesized that after hatching ovotestes could be transformed into ovaries (Zaborski et al. 1982). This hypothesis can now be rejected.

Ovotestes have been described in adult turtles of species exhibiting TSD (*Emys orbicularis [europaea]*: Matthey 1927; *Testudo graeca*: Fantham 1905; *Sternotherus odoratus*: Risley 1934). Although presenting immature oocytes at their surface, these gonads produced spermatozoa showing that they functioned as testes. Therefore, gonadal intersexuality did not hinder the male reproductive function in these animals. Cases with gonads producing both mature oocytes and spermatozoa (true hermaphrodites) have not been described in turtles. Moreover, it seems that intersexuality does not occur to the same degree in all turtle species. It appears to be exceptional in *Trachemys scripta* (Wibbels et al. 1991; Crews et al. 1994), whereas it is frequent in *Emys orbicularis* (Girondot 1993). As suggested by Desvages et al. (1993), these differences could be due to species-specific responses to estrogens involved in either the differentiation of the ovarian cortex or the inhibition of testicular cords. Both processes are under the control of estrogens (Dorizzi et al. 1991). In *Emys orbicularis*, low levels of estrogens are sufficient to induce the differentiation of an ovarian cortex, whereas much higher levels of estrogens are required to inhibit the development of testicular cords. The activity of aromatase, the enzyme that converts androgens to estrogens, has been studied in gonads of embryos incubated at different temperatures. At 25°C (male-producing temperature), aromatase

activity in differentiating testes remains very low from the beginning of the thermosensitive period to hatching. At 30°C (female-producing temperature), aromatase activity increases strongly in differentiating ovaries during the same period (Desvages & Pieau 1992). At 28.5°C (pivotal temperature), aromatase activity in ovotestes is only slightly higher than in testes at 25°C, whereas in ovaries it is somewhat lower than at 30°C (Girondot 1993 and unpublished results). In *Emys orbicularis*, therefore, a slight increase in aromatase activity (thus of estrogens) is sufficient to induce the formation of an ovarian-like cortex at the surface of testes. In *Trachemys scripta*, and possibly some other species, the levels of estrogens for testicular cord inhibition could be close to those needed for differentiation of the ovarian cortex.

Thus, the available evidence indicates that although intersex gonads can be identified from the simultaneous presence of both cortical and medullary structures, these ovotestes will evolve and function as testes. Generally, the oocytes degenerate, but even if a few of them remain on the surface of the gonad, the individual functions as a male.

## Methods for Feminizing Turtle Embryos

Two methods are available to feminize embryos in species with TSD: incubation of eggs at a feminizing temperature, and treatment of eggs with exogenous estrogens ("estrogen-spotting procedure"; Crews et al. 1994). We examine methods and their consequences.

### Feminization by Temperature

The TSD has been shown to occur in experimental and natural conditions, but its importance in natural conditions is not well established and could be different according to the species. If temperature is the sole factor determining sex, then the content of nests should be mainly unisexual. Such a situation has been described in some localities for map turtles (*Graptemys* sp., Vogt & Bull 1984). In marine turtles, however, it is not as frequent as it should be if temperature were the only sex-determining factor (Mrosovsky & Godfrey 1995). It has been demonstrated that a genetic component influences sex determination for incubation at constant temperature within the interval of temperature in which both sexes are produced (Bull et al. 1982; Janzen 1992; Girondot et al. 1994a). Under natural conditions the incubation temperature often fluctuates from male-producing to female-producing temperatures during the thermosensitive period. In such a temperature regime, the genetic component of sex determination could be of major importance (Pieau 1982), although it has not yet been demonstrated how the results would be differentiated from those produced by incubation at pivotal temperature.

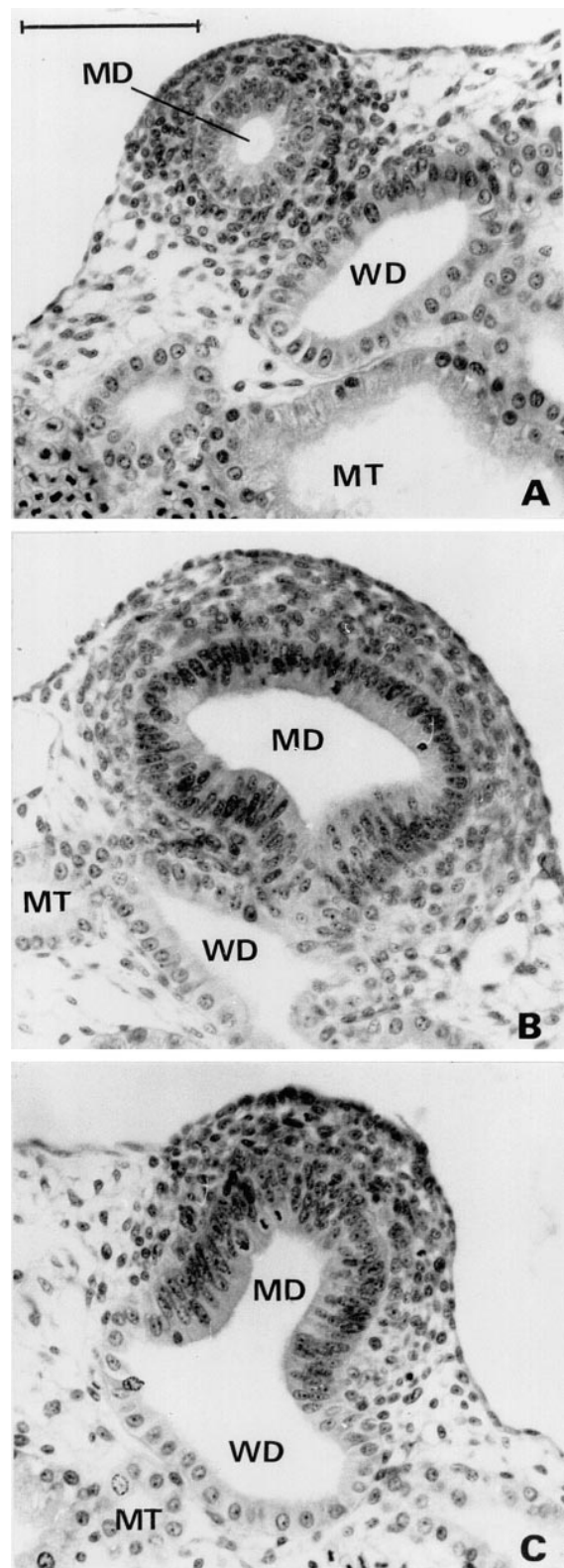
Analysis of H-Y expression in *Emys orbicularis* individuals from natural populations also argues for a major genetic influence in sex determination (Servan et al. 1989; Girondot et al. 1994b). According to these data, natural incubation of more than 80% of eggs would occur in conditions allowing the genetic component to influence sex determination (Girondot et al. 1994b). Such a situation probably occurs more frequently than believed in turtles. Therefore, it is evident that the introduction of many females to a population would change its sexual genetic evolution.

#### Feminization by Estradiol

In reptiles with TSD, the sexual differentiation of gonads is controlled by the level of endogenous estrogens during the thermosensitive period. Even when eggs are incubated at masculinizing temperature, if estradiol or estrone are injected or deposited onto the eggshell, the gonads develop as ovaries and the embryos become females (reviewed by Pieau et al. 1994b).

Several known problems arise with estrogenic treatments. It is impossible to control exactly the amount of estrogen incorporated into the embryo. Moreover, treatments of embryos with estrogens during the thermosensitive period may induce morphological abnormalities of both gonads and genital ducts. Abnormalities of gonads were observed after the injection of high dosages (80–180  $\mu\text{g}$ ) of estradiol benzoate into eggs of *T. graeca* (Pieau 1970) and of low (5–10  $\mu\text{g}$ ) as well as high dosages (50–120  $\mu\text{g}$ ) of this steroid into eggs of *E. orbicularis* (Pieau 1974b). Commonly, the volume of the gonadal medulla was extremely reduced and the cortex was also much thinner than in control ovaries. The post-natal development of such “hypoovaries” has not been studied.

Abnormalities of Müllerian ducts were observed with the high dosages of estradiol benzoate in both *T. graeca* and *E. orbicularis* embryos (Pieau 1969, 1970, 1974b). At the time of the injections, the Müllerian ducts were not complete, and the estrogenic treatment often resulted in the arrest of their lengthening. This was associated with the fusion of the Müllerian epithelium to the Wolffian epithelium, or even with the opening of the caudal end of the Müllerian ducts into the Wolffian ducts. These abnormalities were previously described



**Figure 1.** Transverse sections through Wolffian duct (WD) and Müllerian duct (MD) of *Emys orbicularis* embryos at stage 23, incubated at male-producing temperature (25°C): control, the Müllerian duct has begun to regress, and it is separated from the Wolffian duct by mesenchymal cells (A); treated with 120  $\mu\text{g}$  of estradiol benzoate at stage 20, the caudal end of the Mülle-

rian duct does not reach the cloaca, but its epithelium has proliferated and fused with that of the Wolffian duct (B); treated with 80  $\mu\text{g}$  of estradiol benzoate at stage 20, the caudal end of the Müllerian duct opens into the Wolffian duct (MT, mesonephric tubule) (C). Bar, 50  $\mu\text{m}$ .

and explained in *T. graeca*: they were found in 13 out of 17 embryos treated with 120–180  $\mu\text{g}$  of estradiol benzoate (Pieau 1970). They were also observed in *E. orbicularis* after treatments with 50–80  $\mu\text{g}$  of estradiol benzoate during the thermosensitive period (Pieau 1974b). Figure 1 shows two cases of such abnormalities in *E. orbicularis*. During normal development, Müllerian ducts are terminated by a short cord of large cells that probably play a major role in their craniocaudal growth (Raynaud & Pieau 1985). This cord is destroyed by estrogenic treatment (Pieau 1970; Raynaud & Pieau 1985). Therefore, even if Müllerian ducts did not open into Wolffian ducts, they probably would not produce complete oviducts in adults; even if the ovaries produced mature oocytes, fertilization would not be possible, and the unfertilized eggs would not be evacuated from the oviducts. As a consequence, not only would the reproductive female function be impossible, but the animals would be exposed to internal injuries if the calcareous eggshell were formed.

The use of exogenous estradiol for turtle feminization should be avoided for other reasons. In natural development, not all tissues are equally in contact with the steroid. In *Emys orbicularis*, for example, slight aromatase activity can be detected within the adrenal-mesonephros complex, whereas in the adjoining ovary there is high aromatase activity (Richard-Mercier et al. 1995). When estradiol is deposited onto the eggshell, however, all tissues are subjected to high doses of this hormone. We do not know all the physiological and behavioral consequences of such an early estrogen impregnation of tissues, although long-term studies of the leopard gecko (a lizard with TSD) indicate that the fecundity of estrogen-determined females (treated with low dosage of estrogens) is indistinguishable from that of temperature-determined females (Crews et al. 1994).

### Simulation of Long-Term Effects of a Conservation Plan

The evolution of primary sex ratio (proportion of males at the end of the thermosensitive period for sex determination) should follow a frequency-dependent selection (Charnov 1982). If males are rare in a population, each male will contribute, on average, genetically more to the next generation than each female because all individuals have one mother and one father. Thus, individuals becoming males will be favored over females; as a consequence, masculinizing alleles will be selected for. In the same way, feminizing alleles would be favored if females were rare. Thus, the primary sex ratio would stabilize to an equilibrium value, and all deviation in favor of one sex would select alleles determining the other sex. The particular value of equilibrium is 0.5 if the Fisherian assumptions are retained, but it can be different in the

Charnov-Bull model for the evolution of TSD (Charnov 1982). In the case of manipulation of the primary sex ratio by artificial incubation at feminizing temperatures or by estrogen treatment, masculinizing alleles should be favored.

We test the demographic effects of this hypothesis by a simulation of the dynamics of a population of turtles in which the primary sex ratio has been manipulated over a 30-year period by a conservation plan favoring the production of females in the ratio proposed by Vogt (1994).

A population of approximately 1,000 individuals is simulated during 10,000 years for stabilization of the population structure (years  $-10,000$  to 0). The annual survival at adult stage is 0.95, with no differences between males and females. Reproductive maturity is reached at 10 years for both sexes. During the stabilization process, the number of embryos surviving per adult female (reproductive output per female) is calculated every year to maintain a constant population size. The average reproductive output per female calculated for the years  $-1,000$  to 0 will be used after year 0. In this case, the population size remains constant from year to year, independent of the initial population size, if no other perturbations are introduced.

Sex determination in the model includes the biochemical knowledge of this process (Pieau et al. 1994b). Differentiation of testis occurs if estrogens within the gonad remain low; otherwise the gonad differentiates as an ovary. The amount of estrogens within the gonad is regulated via the aromatase enzyme by both temperature (low level at masculinizing temperature and high level at feminizing temperature) and by genetic polymorphism at a locus controlling the activation of aromatase by temperature. This genetic polymorphism is defined by the allelic composition at one locus (individuals are diploid). At year  $-10,000$  heterozygosity is 1 at this locus because alleles are attributed by random numbers and therefore sexual genotype is a quasi-continuous character. At the end of the stabilization process only a few alleles are retained. Each year, a value defining the global tendency of annual incubation temperature is calculated by a Gaussian random number. For each egg the incubation temperature is calculated by a Gaussian random number based on the tendency of the current year. As a result the sexual phenotype for each embryo is the result of a calculation taking into account its sexual genotype and incubation temperature (low temperatures are masculinizing and higher ones are feminizing). The probability of a mutation by a transmitted allele in 1 year is  $3 \times 10^{-5}$ .

We examined two cases. In the first the probability of having offspring was independent of the sex and incubation conditions of the adult. Therefore, all the Fisherian conditions (Fisher 1929) were retained, and consequently the primary sex ratio was 0.5. In the second case, the probability of having offspring was enhanced for females according to their own incubation tempera-

ture and decreased by the same amount for males; so TSD was favored over GSD (Charnov 1982). Because the fitness effect is the inverse for males and females according to their incubation temperature, the equilibrium primary sex ratio was still 0.5.

After the stabilization process, a conservation plan worked on the population for 30 years. Annually, 95% of the eggs were feminized; the remaining eggs were masculinized. After that, the population was left on its own with the same parameters as during the 10,000 years of stabilization. Based on the reproductive output estimated from years  $-1,000$  to  $0$ , changes in the population size would therefore be due to the effect of feminization. Different conservation protocols were simulated, and the number of individuals in the population were taken as a measure of the effectiveness of the conservation plan. As a control, identical populations were simulated without the conservation plan. Twenty sets of managed and control populations were computed for each of the studied protocols. The mean numbers of adults in the population from year 31 to 230 and year 231 to 2030 were compared by two-tail paired  $t$  test. All the tests were performed by means of Statview 4.02 (abacus concept) on an Apple Macintosh.

### Effects of the Conservation Plan on Population Dynamics

Figure 2 shows the results obtained by using the Fisherian conditions. Before the conservation plan, the primary sex ratio (hatchling male frequency) fluctuated slightly, with the proportion of males staying close to 0.5. The sex ratio of adults fluctuated less because it is a weighted mean of the primary sex ratio, derived over a period of more than 10 years. When the conservation plan began, the hatchling male frequency fell immediately to approximately 0.05. The population sex ratio fell gradually until it was highly feminized at the end of the 30 years of the conservation plan (population male frequency of 0.11). After the plan the hatchling male frequency went up immediately to the value of 0.55–0.7, showing that masculinizing alleles had been selected. The population male frequency increased slowly to 0.5–0.6. These values were reached at about 70 years. The mean hatchling male frequency 2000 years after the period of feminization was 0.54, which shows a long-term effect of masculinization. In the control population, the male frequencies stayed around 0.5. The number of individuals in the population was enhanced by the feminization process and reached the maximum of 1923 individuals at year 60. Therefore, this process permits rapid doubling of the population size. Because females have male-biased offspring, however, the population size soon decreased and finally became smaller than the control population.

Statistical tests were performed on a set of 20 populations because the control population exhibited changes in its size simply due to random fluctuations. The mean number of individuals in the population during years 31–230 (Fig. 3) was significantly higher for the feminized population (Fig. 3C, Fisherian model) than for the control population (Fig. 3A, Fisherian model) (two-tailed paired  $t$  test,  $t = 11.66$ ,  $df = 38$ ,  $p = 0.0001$ ), indicating that the feminization process produced a short-term enhancement of the population size. This is what Vogt (1994) calls “the boost effect.” But, the mean number of individuals in the population during years 231–2030 was significantly lower for the feminized population (Fig. 3C, Fisherian model) than for the control population (Fig. 3A, Fisherian model; two-tailed paired  $t$  test,  $t = -2.128$ ,  $df = 38$ ,  $p = 0.04$ ), indicating that the feminization process produced a long-term deleterious effect on the population size. The average deficit of the number of individuals in the feminized population compared to the control population for years 31–2030 was 28%. It should be noted, however, that two feminized populations out of the 20 exhibited higher juvenile production than the control did.

The deleterious effect was not observed in the population simulated with the Charnov-Bull conditions for the evolution of TSD. The number of individuals in the femi-

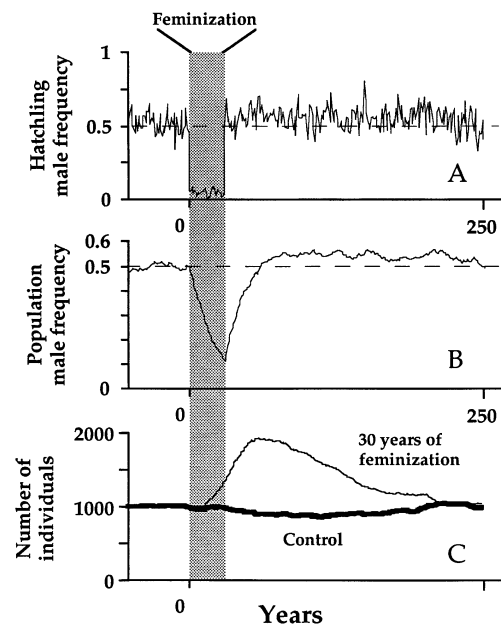
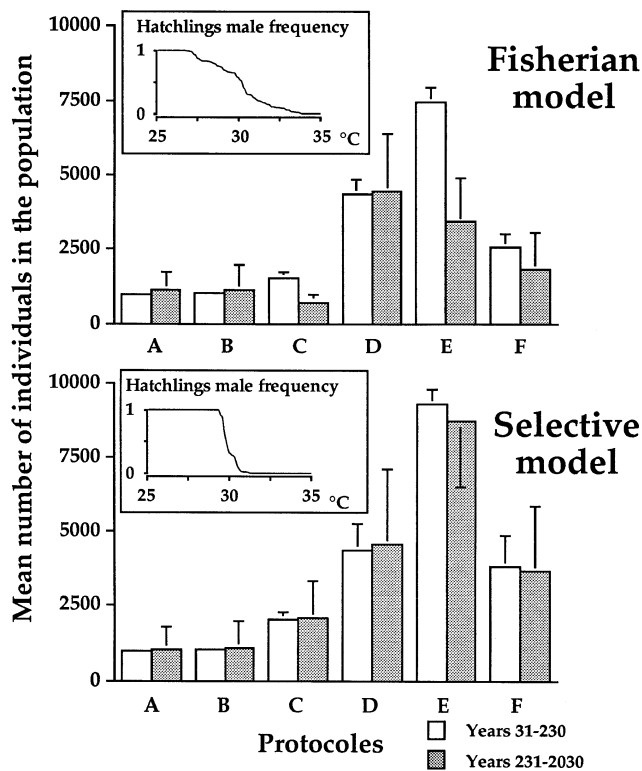


Figure 2. Evolution of a population over 250 years after the action of a conservation plan feminizing 95% of the turtle embryos for 30 years. The plan begins at year 0. At year 30 the population is no longer manipulated to allow evaluation of the consequences of feminization on the hatchling male frequency (A), the population (adults + juveniles) male frequency (B), and the number of individuals in the population (C).



**Figure 3.** Mean number of individuals (adults + juveniles) over the 199 first years (white bars) and the 1799 following years (gray bars) for Fisherian or selective conditions. The protocols are control population, no feminization (A); 5% of the eggs are feminized, other eggs are untouched (B); 95% of the eggs are feminized, other eggs are masculinized (C); 50% of the juveniles with first annual survivorship two times greater than untouched ones (D); 50% of the eggs are managed and among them feminization occurs at a 95% level, the remaining 5% being masculinized. Managed juveniles have a first annual survivorship two times greater than those from untouched eggs (E); (F) same as C but annual survival is 0.85. In the two graphs in the boxes, the male frequency among newborns for incubation temperatures from 25° to 35°C is shown.

nizing population remained higher even in the long-term (Fig. 3A and 3C for selective model; years 31–230:  $t = 17.36$ ,  $df = 38$ ,  $p = 0.0001$ ; years 231–2030:  $t = 3.30$ ,  $df = 38$ ,  $p = 0.002$ ). Genetic variability of sex determination was much more reduced in selective conditions than in Fisherian ones because each time an individual differentiated to the “wrong” sex according to its incubation temperature, the carrying alleles were counterselected. This effect can be seen in the sex determination profile from 25° to 35°C (boxes in Fig. 3) in which the transition from male to female temperature was sharper in the selective model than in the Fisherian one. Also,

the heterozygosity at the sex-determining locus at year 0 was significantly higher in the Fisherian conditions than in the selective ones (0.85 versus 0.68;  $t = 6.80$ ;  $df = 38$ ,  $p < 0.0001$ ). Therefore, the populations simulated with the selective model lacked the variability on the sex-determining gene to respond rapidly to the feminization protocol. The mutation probability of  $3 \times 10^{-5}$  appeared to be insufficient to induce the invasion of masculinizing alleles before the feminization protocol is stopped.

#### Effect of Longevity

The feminization protocol was carried out in 20 populations exhibiting an annual survival of 0.85 (Fig. 3F) and compared with ones exhibiting an annual survival of 0.95 (Fig. 3C). The feminization protocol produced better results for annual survival of 0.85 than for 0.95 in both selective and Fisherian models (mean number of individuals in the population from years 31 to 2030: 2069 versus 3677,  $t = -3.05$ ,  $df = 38$ ,  $p = 0.0041$ ; 974 versus 1652,  $t = -2.57$ ,  $df = 38$ ,  $p = 0.01$ ). The deleterious effect of feminization observed in Fisherian conditions is significantly stronger because the longevity is higher (test for years 231–2030: two-tailed paired  $t$  test,  $t = -3.07$ ,  $df = 38$ ,  $p = 0.003$ ). Because turtle species with TSD exhibit higher survival than those with genotypic sex determination (Bull & Bulmer 1989; Janzen & Paukstis 1991), populations with TSD are very sensitive to the feminization process.

#### Effect of Female Frequency among Neonates

The female frequency in neonates was the parameter directly selected. We tested the effect of the introduction of a low percentage of females on the evolution of a population (5% of the eggs were feminized and the other eggs were left in natural conditions) compared to a control population without any change. No difference in the mean number of individuals in the population was observed in either kind of populations (Fig. 3B; Fisherian conditions:  $t = 0.16$ ,  $df = 38$ ,  $p = 0.8$ ; selective conditions:  $t = 0.26$ ,  $df = 38$ ,  $p = 0.8$ ). Thus, if only 5% of the eggs were managed, neither the boost effect nor the deleterious effect of feminization were observed. Selection for masculinizing alleles was not strong if the conservation plan displaced the sex ratio only slightly from the equilibrium value (Bull & Charnov 1988).

#### Effect of Survival of the Managed Hatchlings and Juveniles

To test for a potential effect of survival enhancement in the managed eggs (i.e., headstarting), two new simulations were performed. In the first simulation, 50% of the eggs were managed, and feminization occurred at a 95% level among them; survivorship for the first year was

two times higher for individuals from the managed eggs (Fig. 3E). In the second simulation, 50% of the eggs were protected but not feminized; survivorship for the first year was two times higher for the juveniles from the managed eggs (Fig. 3D). The deleterious effect of feminization was overcome by headstarting (compare protocols C and E in Fig. 3). But, headstarting without feminization was superior to feminization in Fisherian condition (comparison between protocols D and E for years 231–2030:  $t = -2.17$ ,  $df = 38$ ,  $p = 0.03$ ), whereas the deleterious effect was not observed in selective conditions (comparison for protocol E between years 31–230 and 231–2030:  $t = -1.36$ ,  $df = 38$ ,  $p = 0.17$ ). It should be noted, however, that headstarting alone is not a good conservation tool for long-lived species because it cannot overcome the predation of adults (Heppell et al. 1996).

### Effect of the Number of Years of Management and Population Size

Because turtles are long-lived vertebrates, individuals continue to reproduce for many years. Therefore, the effect of the number of years of management could be treated exactly as the percentage of feminization in the neonates. If the number of years of management is low, only a low percentage of feminized individuals will effectively contribute to adults, and therefore neither the boost effect nor the deleterious effect of feminization will be observed. But if the number of years of management is large, both boost and deleterious effects can be observed according to the level of feminization.

The results of studies of population genetics show that random fluctuation of gene frequency (genetic drift) will be important if the size of the population is very reduced. The deleterious effect observed in the simulation can thus be enhanced for very small populations, say less than 30 animals. In fact, conservation plans are often established as a priority for decreasing or small populations.

### Effect of the Type of Genetic Variation of Sex Determination

The type of genetic variation involved in sex determination is essential for estimating the inertia of the return to equilibrium sex ratio and for the potentiality to respond to the sex ratio manipulation. If sex determination is purely environmental, then feminization cannot induce the selection of masculinizing alleles because of the lack of genetic variability. If a genetic component influences sex determination, two major sex-determining mechanisms can be considered: monolocus and multilocus. The heritability of sex ratio is high for turtles when incubation is performed within the range of temperatures producing both sexes (Bull et al. 1982; Janzen 1992), indicating that only a few loci are involved in the genetic

component of sex determination. Thus, the monolocus mechanism for sex determination used in the simulation is plausible. Without exact knowledge of all parameters involved in sex determination, however, it is difficult to predict how the sex-reversed individuals will affect the equilibrium sex ratio. To date, these parameters are unknown even for the best-studied species, such as *Emys orbicularis* or *Trachemys scripta*. Moreover, it is possible that the phenomenon of sex determination in each population varies with both time and space.

## Conclusions

Considering a turtle species with both genotypic (ZZ/ZW) and temperature-dependent sex determination, Mrosovsky (1994) observed that “production of almost all females in a hot year will sow the seed of a corrective masculinization in later years.” The evolution of a balanced sex ratio (1 male:1 female) by the process of frequency-dependent selection of the minority sex was experimentally demonstrated in the fish *Menidia menidia*, a species with genetically based variations in TSD (Conover & Van Voorhees 1990). In the simulations we have performed here, the artificial feminization of turtle embryos in a population results in the selection of masculinizing alleles. If the masculinizing alleles were already present in the population at the beginning of the 30-year conservation plan, their frequency will simply be enhanced, and the pivotal temperature will be shifted to male-producing temperature. This case is observed in the Fisherian model simulations (Fig. 3, box, Fisherian model). Consequently, a long-term increase in the proportion of males in the population is observed. If the size of the population is restricted—for example, by food supply or nesting space—the number of females also will be reduced compared to a control population without feminization. Consequently, the number of females could not be sufficient to ensure the persistence of the population (Girondot & Picaud 1996). We have shown, however, that an a priori effect cannot be anticipated because it depends on unknown parameters. Therefore, only the a posteriori effect can be noticed: either the population size decreases or it does not. This is playing to the sorcerer’s apprentice.

On the other hand, if masculinizing alleles are not present in the population at the time of feminization by conservation plan, these alleles can be acquired only by mutation. This case is observed in simulations in the selective model because strongly feminizing or masculinizing alleles have been counterselected during the stabilization process (Fig. 3, box, selective model). But acquisition of a masculinizing allele by mutation takes a much longer time than the 30 years of a conservation plan. Finally, this acquisition is not observed and the pivotal temperature is not changed. Consequently, no long-



term change in sex ratio is observed in the selective model.

In the procedure using exogenous estrogens, the estrogen-feminized embryos become females, whereas they could have been incubated at a male-producing temperature. In the Charnov-Bull model for the evolution of TSD (Charnov 1982), these females will be less fit than females incubated at normal female-producing temperature. Moreover, we have seen that “hypoovaries” can be obtained, even with low dosages of hormone, and it cannot be assumed that such gonads would function normally in adults. Abnormalities in Müllerian ducts have been observed with relatively high doses of hormone in *E. orbicularis* and *T. graeca*. But the threshold below which they never occur has not been determined. Moreover, there are likely to be different responses for each species, state of development, and possibly different environmental parameters. Likewise, information on the reproductive capacities and behavior of estrogen-induced females as adults are needed. Experiments on these aspects are in progress for *T. scripta* (Crews et al. 1994).

Vogt (1994) recommended avoiding incubation at pivotal temperature to avoid production of intersexes, but this is probably not a problem. First, although intersexuality occurs frequently in some species such as *E. orbicularis*, it seems rare in others such as *T. scripta* (Crews et al. 1994). Second, intersexuality is a transient phenomenon because ovotestes develop into testes after hatching. Even though some oocytes are maintained at the surface of the testes, they will not mature, and spermatozoa will be produced. Third, the incubation of eggs around the pivotal temperature is probably more frequent than believed in natural nests.

For all these reasons, we think that rather than manipulating sex ratios, conservation plans for reptiles should focus on the protection of the animals themselves and their habitats and nesting sites. In addition to providing protection against predators and human exploitation, this would allow freshly laid eggs that are clearly exposed to risk to be transplanted within the nesting sites rather than into hatcheries.

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