

Temperature sex-reversal in amphibians and reptiles

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ABSTRACT The sexual differentiation of gonads has been shown to be temperature-sensitive in many species of amphibians and reptiles. In two close species of salamanders, *Pleurodeles poireti* and *P. waltl*, both displaying a ZZ/ZW mechanism of genotypic sex determination (GSD), the rearing of larvae at high temperatures (30° - 32°C) produces opposite effects: ZZ genotypic males of *Pleurodeles poireti* become phenotypic females whereas ZW genotypic females of *P. waltl* become phenotypic males. Sex-reversal of these individuals has been irrefutably demonstrated through genetic, cytogenetic, enzymatic and immunological studies. In many turtles, both sexes differentiate only within a critical range of temperature: above this range, all the individuals become phenotypic females, whereas below it, 100% become phenotypic males. The inverse occurs in some crocodiles and lizards. In many species of these three orders of reptiles, females are obtained at low and high temperatures, and males at intermediate ones. Preliminary studies in turtles (*Emys orbicularis*) indicate that within the critical range of temperature, sexual phenotype conforms with GSD, but that above and below this range, GSD is overridden. Temperature shifts during larval development in salamanders and during embryonic development in reptiles allowed the determination of thermosensitive stages for gonadal differentiation. Estrogens synthesized in the gonads at these stages appear to be involved in their sexual differentiation, higher levels being produced at feminizing temperatures than at masculinizing ones. The phenomenon of temperature sensitivity of gonadal differentiation occurs in species showing a very early stage in the evolution of sex chromosomes. Its adaptive value, chiefly in reptiles, remains an open question.

KEY WORDS: *amphibians, reptiles, sex determination, gonadal differentiation, temperature sensitivity, estrogens*

Introduction

In 1898, Hertwig observed that high rearing temperatures accelerate the developmental rate of amphibian larvae. The study of the effects of temperature on sexual differentiation was then undertaken by Witschi in *Rana temporaria* and in *Rana sylvatica* (Witschi, 1914, 1929). Other anuran species (*Bufo vulgaris*, Piquet, 1930; *Rana japonica*, Yoshikura, 1959, 1963; *Rana catesbeiana*, Hsü *et al.*, 1971) and a urodele species (*Hynobius retardatus*, Uchida, 1937a, b) were then examined. In all cases, histological modifications of gonads and biased sex ratios were described. However, the complete and functional sexual inversion of gonadal phenotype was not demonstrated, as there was not any known marker making possible the identification of sexual genotype of the individuals, and because the animals were reared until only a few weeks after metamorphosis, their progeny were not studied. Moreover, the interpretation of the results obtained in these species is not easy

because of the existence of different sexual races in amphibians.

In sexually differentiated races, such as *Rana sylvatica* (Witschi, 1929), *Rana japonica* (Yoshikura, 1959, 1963), *Rana catesbeiana* (Hsü *et al.*, 1971) and *Bufo vulgaris* (Piquet, 1930), the gonads directly evolve according to the sexual genotype at room temperature, and sex ratio is balanced at metamorphosis.

In sexually undifferentiated races, such as probably *Rana temporaria* (Witschi, 1914; Piquet, 1930), the sexual differentiation of the gonads in genotypic males presents a transient period of feminization. At metamorphosis, all individuals appear to have ovaries. Then a phase of inter-sexuality characterizes male development, and the sex ratio becomes balanced several weeks and even several months after metamorphosis.

Abbreviations used in this paper: GSD, genotypic sex determination; SD-H-Y, serologically detectable antigen H-Y; TSD, temperature-dependent sex determination.

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In sexually semi-differentiated races, such as *Hynobius retardatus* (Uchida, 1937a, b), phenotypic females and intersexes are observed in variable proportions at metamorphosis. Then, intersexes and some phenotypic females will evolve towards phenotypic males, the feminizing period being shorter than in undifferentiated races.

Depending on the sexual race, intersexuality and subsequently sex ratio will evolve differently during the juvenile period in amphibians. In undifferentiated and semi-differentiated races, intersexuality may indeed correspond to the transient stage of gonadal differentiation in genotypic males, whereas in differentiated races, it may result from partial inversion of gonadal phenotype in genotypic females. Therefore, in studying the influence of temperature on gonadal differentiation, it will be difficult in some cases to distinguish the effects of temperature from the normal evolution of gonads. Moreover, it is generally accepted that intersexes will evolve towards phenotypic males. Thus, considering the partial masculinization of genotypic females at metamorphosis, it has often been assumed that in amphibians, the rearing of larvae at high temperatures leads to 100% males. Nevertheless, the data obtained in two species of Urodeles, *Hynobius retardatus* (Uchida, 1937a,b) and *Pleurodeles waltl* (Dournon, 1981; Houillon and Dournon, 1986) have shown that intersexes can also evolve towards phenotypic females.

All these considerations and the results obtained in reptiles led two of the authors to reexamine the problem of temperature sensitivity of sexual differentiation in amphibians, and to try to demonstrate without any ambiguity the phenomenon of temperature sex-reversal. The salamander *Pleurodeles waltl*, commonly used in the laboratory, was first chosen (Houillon and Dournon, 1978; Dournon and Houillon, 1983, 1984). Then, a very close species, *P. poireti*, was also studied (Dournon et al., 1984). These two salamanders are from sexually differentiated races and can be hybridized.

In reptiles, the first observation suggesting that the incubation temperature of eggs could influence the hatchling sex ratio was made by Charnier (1966) in a lizard of North Africa, *Agama agama*. In 1971, Pieau obtained biased sex ratios among embryos of two species of turtles, *Emys orbicularis* and *Testudo graeca* and demonstrated in 1972 that they were due to the incubation temperature of eggs, with phenotypic males produced at low temperatures and phenotypic females at higher ones. In 1976, Yntema described another pattern of temperature sensitivity in the snapping turtle *Chelydra serpentina*, females being obtained at low and high temperatures and males at intermediate ones. Then, temperature was found to influence sexual differentiation in 4 other Emydidae (Bull and Vogt, 1979) and in a marine turtle, *Caretta caretta* (Yntema and Mrosovsky, 1979, 1980), but incubation of eggs at different temperatures did not significantly modify sex ratio in *Trionyx spiniferus* (Bull and Vogt, 1979; Vogt and Bull, 1982). In 1980, Bull published the first review on sex determination in reptiles and distinguished species with genotypic sex determination (GSD) from species with temperature-dependent sex determination (TSD). Although questionable (see below), this distinction was subsequently used by many authors. Temperature sensitivity of gonadal differentiation was shown in other marine turtles (Miller and Limpus, 1980; Morreale et al., 1982; Mrosovsky, 1982; Wood and Wood, 1982; Mc Coy et al., 1983; Rimblot et al., 1985), in crocodiles (Ferguson and Joanen, 1982; Webb et al., 1983; Webb

and Smith, 1984) and in lizards (Wagner, in Bull, 1980; Tokunaga, 1985). Many other reviews have been written on the subject (Bull, 1983; Pieau, 1985; Raynaud and Pieau, 1985; Standora and Spotila, 1985; Gutzke, 1987; Deeming and Ferguson, 1988, 1989).

The aim of this paper is to show the importance of comparisons between amphibians and reptiles in understanding the mechanisms involved in temperature sex-reversal. After describing the different patterns of temperature sensitivity of sexual differentiation, we will present successively, the data giving evidence of temperature sex-reversal or of interaction of temperature with genotypic sex determination, and the experiments making possible the delimitation of thermosensitive periods and preliminary data on the involvement of sex steroid hormones in gonadal differentiation. Much of the data have been obtained in our laboratories, respectively in *Pleurodeles waltl* and *P. poireti* for amphibians, in *Emys orbicularis* for reptiles.

Different types of response to temperature for sexual differentiation

Amphibians

As a general rule in amphibians, rearing of larvae at ambient temperature (16° to 24°C) leads to a 1 male : 1 female sex ratio, whereas at extreme warm and cold temperatures compatible with normal development, gonadal differentiation is perturbed in one genotypic sex and the sex ratio may be biased.

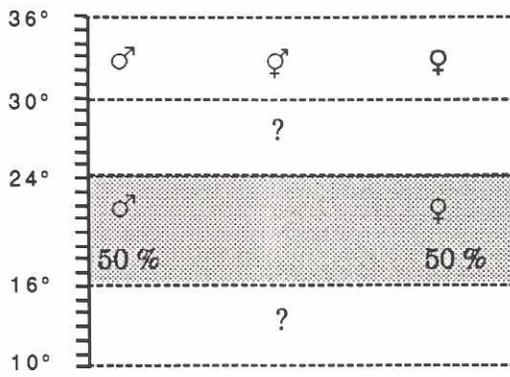
Effects of heat treatment

Witschi (1929) reared at 32°±2°C, for 15 to 33 days, larvae of *Rana sylvatica* from a sexually differentiated race. As treatment started, the gonads had begun to differentiate and the sex ratio was balanced (15 males – 13 females). At the end of treatment, out of 115 individuals, 62 were phenotypic males and 53 displayed transformed – more or less masculinized – ovaries. Although this experiment showed a masculinizing effect of high temperatures, it did not result in 100% phenotypic males. The following experiments performed in anurans (Piquet, 1930; Yoshikura, 1959, 1963; Hsü et al., 1971) and in a urodele species (Uchida, 1937b) corroborated these observations.

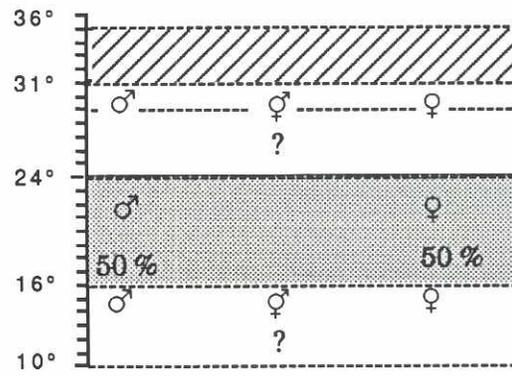
More recently, the effects on sexual differentiation of rearing temperature during larval life have been studied in two close species of salamanders, *Pleurodeles poireti* and *P. waltl*. Both display a ZZ/ZW mechanism of genotypic sex determination. In *P. poireti*, the male homogamety ZZ and the heteromorphism of lampbrush Z and W sex chromosomes were shown after treating larvae with estradiol benzoate (Lacroix, 1970). In *P. waltl*, the male homogamety ZZ was demonstrated by the offspring analysis of individuals treated with estradiol benzoate (Gallien, 1951), and the female heterogamety ZW by embryonic grafts (Collenot, 1973).

The effects of temperature were studied on larvae of *P. poireti* issued from a standard cross ZZ male x ZW female (Table 1). Rearing of larvae at room temperature yielded a balanced (1 male: 1 female) sex ratio. At 30°C, the sex ratio was significantly biased towards females indicating that ZZ genotypic males had become functional phenotypic females (ZZ thermoneofemales). Moreover, some intersexes presenting both ovaries and testes were also obtained (Table 1 and Fig. 1).

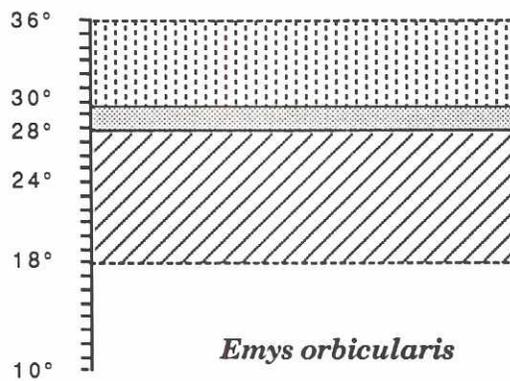
In *P. waltl*, the influence of temperature on sexual differentiation



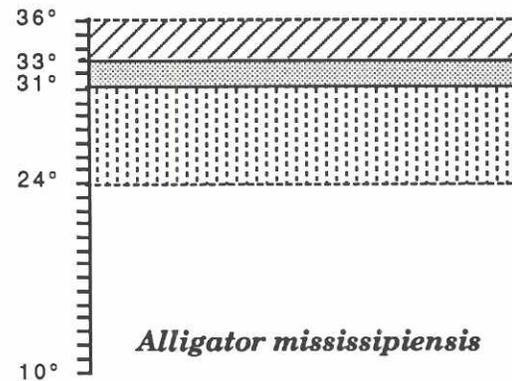
Pleurodeles poireti



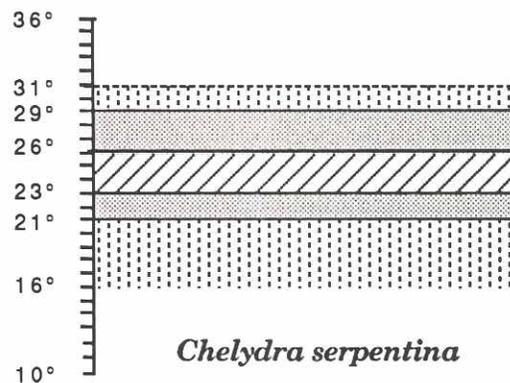
Pleurodeles waltl



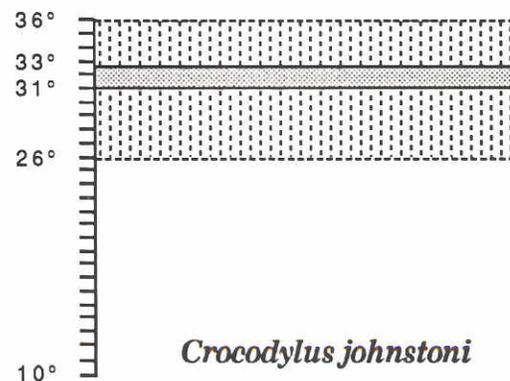
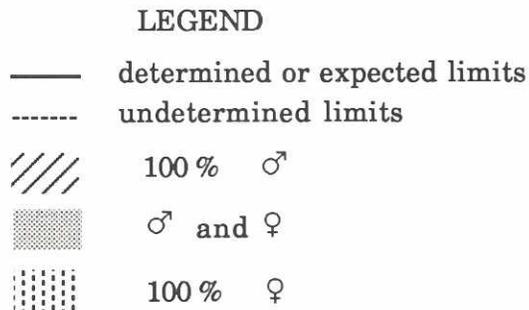
Emys orbicularis



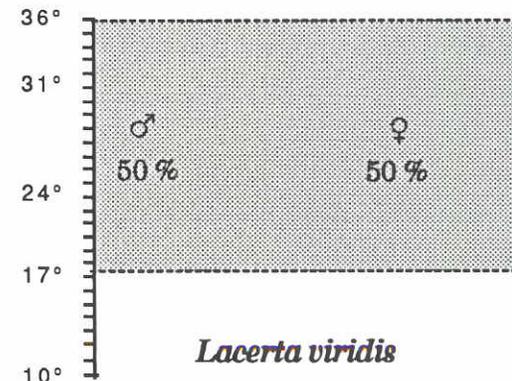
Alligator mississippiensis



Chelydra serpentina



Crocodylus johnstoni



Lacerta viridis

Fig. 1. Different types of response to temperature for sexual differentiation of the gonads in amphibians (*Pleurodeles poireti* and *P. waltl*) and reptiles (other species).

TABLE 1

EFFECTS OF HEAT TREATMENT ON SEXUAL DIFFERENTIATION IN LARVAE OF *PLEURODELES POIRETI* ISSUED FROM A CROSS ZZ STANDARD MALE x ZW STANDARD FEMALE

Temperature	Number and percentage			Total
	Males	Intersexes	Females	
20° ± 2°C (room)	22 52.4 % (50 %)	0	20 47.6 % (50 %)	42
30°C	8 22.9 %	4 11.4 %	23 65.7 %	35

The theoretical sex ratio in conformity with genotypic sex determination is indicated in parentheses.

was examined on larvae from two types of crosses: 1) ZZ standard males x ZW standard females (Table 2), and 2) ZZ standard males x WW thelygenous females (Table 3). At room temperature, the observed sex ratios were in accordance with the theoretical sex ratios, respectively 50% males – 50% females and 100% females (Tables 2 and 3, and Fig. 1). At 30°C, among individuals from crosses of type 1, sex ratios were biased in favor of males and intersexes with testes and ovaries were also found (Table 2). Among individuals from crosses of type 2 (all ZW genotypic females), males and intersexes differentiated (Table 3). At 32°C, in type 1 as well as in type 2 crosses, all animals became phenotypic males (Tables 2 and 3, and Fig. 1). Therefore, ZW genotypic females became functional phenotypic males ("ZW thermoneomales") and there was no difference in the mortality rates of ZZ and ZW individuals.

These results show that warm temperatures have the opposite effects in *P. poireti* and *P. waltl* (Fig. 1), complete or partial sexual inversion affecting ZZ genotypic males in the first species and ZW genotypic females in the second species (Dournon and Houillon, 1984, 1985).

Effects of cold treatment

Witschi (1914) and Piquet (1930) maintained larvae of *Rana temporaria* at 10° – 12°C and obtained an excess of juvenile phenotypic females. Unfortunately, the sexual race of this species

TABLE 2

EFFECTS OF HEAT TREATMENT ON SEXUAL DIFFERENTIATION IN LARVAE OF *PLEURODELES WALTL* ISSUED FROM CROSSES ZZ STANDARD MALES x ZW STANDARD FEMALES

Temperature	Number and percentage			Total
	Males	Intersexes	Females	
20° ± 2°C (room)	617 49.1 % (50 %)	0	639 50.9 % (50 %)	1256
30°C	229 70 %	22 7 %	76 23 %	327
32°C	320 100 %	0	0	320

The theoretical sex ratio in conformity with genotypic sex determination is indicated in parentheses.

was not defined. It was probably undifferentiated.

In *P. waltl*, larvae are difficult to rear at low temperatures since they are sensitive to mycoses and epizootis. Moreover, the developmental rate is extremely reduced. Thus, at 20°±2°C, the larval period extending between stages 42 and 50 lasts approximately 25 days, whereas at 15°C, it lasts 2 to 3 months depending on the individual. A juvenile intersex with both testes and ovaries was obtained among animals from a standard off spring reared at 15°C between these stages (Table 4, and Fig. 1). This animal could be a feminized ZZ genotypic male. However, there is not yet evidence of obtention of fertile ZZ thermoneofemales in *P. waltl*.

Reptiles

Sex ratio as a function of the incubation temperature of eggs has been established, generally at hatching, in many species of reptiles and has allowed the definition of 5 types of response to temperature for sexual differentiation of the gonads (Fig. 1).

Pattern 1: type *Emys orbicularis*

TABLE 3

EFFECTS OF HEAT TREATMENT ON SEXUAL DIFFERENTIATION IN LARVAE OF *PLEURODELES WALTL* ISSUED FROM CROSSES ZZ STANDARD MALES x WW THELYGENOUS FEMALES

Temperature	Number and percentage			Total
	Males	Intersexes	Females	
20° ± 2°C (room)	0	0	847 100 % (100 %)	847
30°C	21 44 %	5 10 %	22 46 %	48
32°C	280 100 %	0	0	280

The theoretical sex ratio in conformity with genotypic sex determination is indicated in parentheses.

Both sexes are obtained only within a critical range of temperature. Above this range, all the individuals become phenotypic females whereas below it, 100% become phenotypic males. In *E. orbicularis*, the incubation of eggs from several clutches at different temperatures gave, around hatching (Pieau, 1976): 100% phenotypic males below 27.5°C; phenotypic males and intersexes (with ovotestes) between 27.5° and 28°C; males, females and intersexes between 28° and 29°C; a majority of females and a low percentage of intersexes between 29° and 29.5°C; 100% phenotypic females above 29.5°C. After hatching, intersexes evolve towards phenotypic males (unpublished results). Therefore, it may be considered that in *E. orbicularis*, all the individuals become phenotypic males below 28°C and phenotypic females above 29.5°C. Both sexes may differentiate between 28° and 29.5°C. However, within this narrow transitional range, the progeny of some wild individuals are unisexual (Zaborski *et al.*, 1988 and unpublished results).

This pattern has been shown in 7 species of turtles and may occur in 9 more (Ewert and Nelson, personal communication).

Pleurodeles waltl

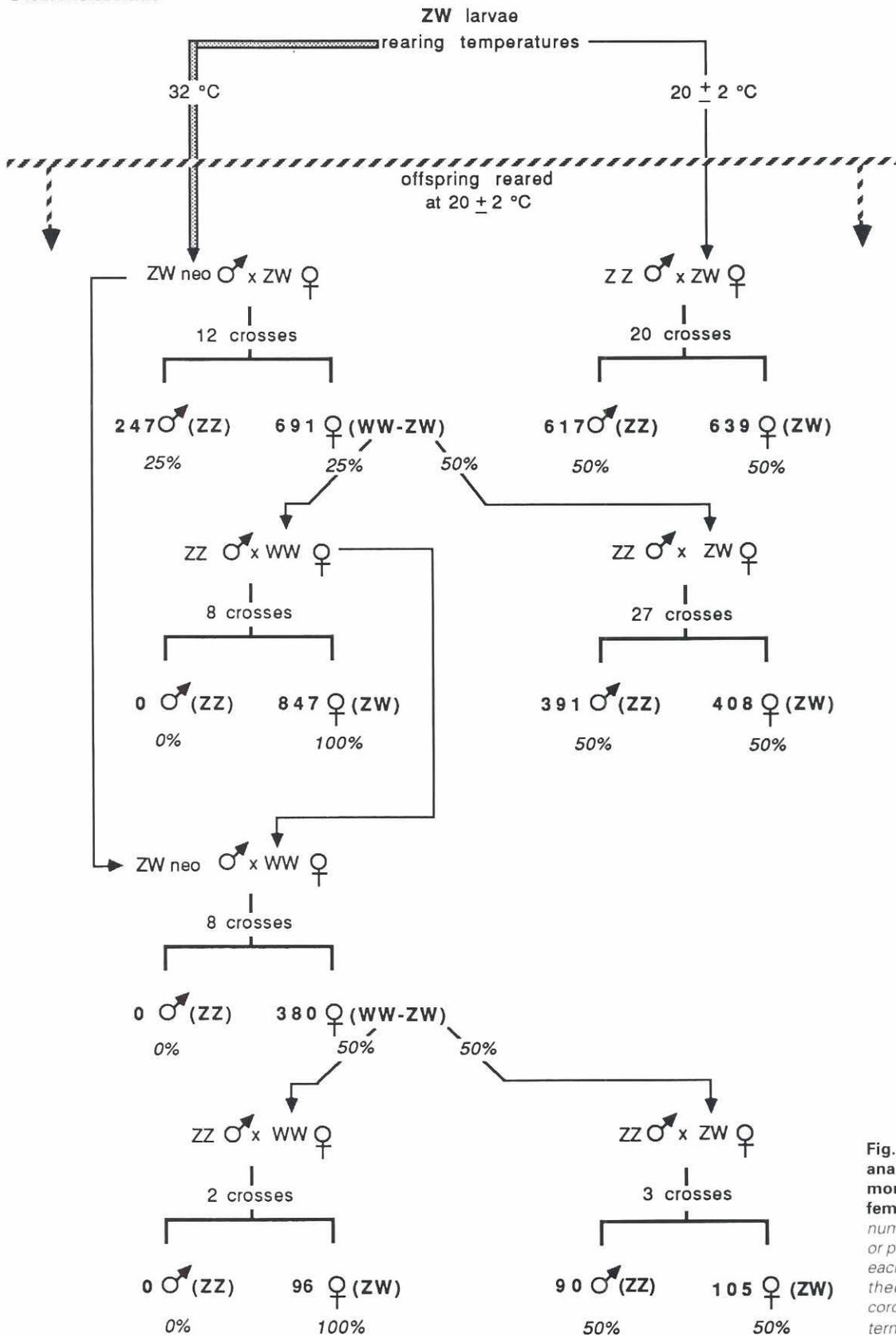
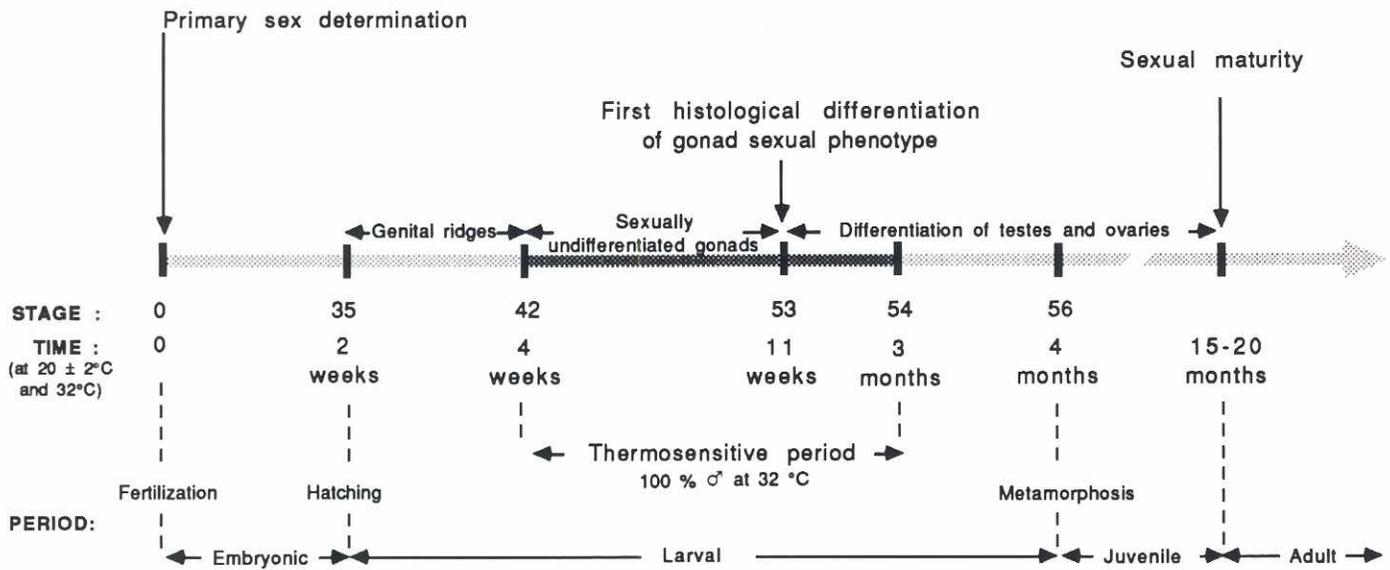


Fig. 2. Sex ratio and genetic analysis in offspring of ZW thermoneomales and ZW standard females of *Pleurodeles waltl*. The number of phenotypic males and/or phenotypic females obtained in each series of crosses, and the theoretical percentages in accordance with genotypic sex determination are indicated.

Pleurodeles waltl



Emys orbicularis

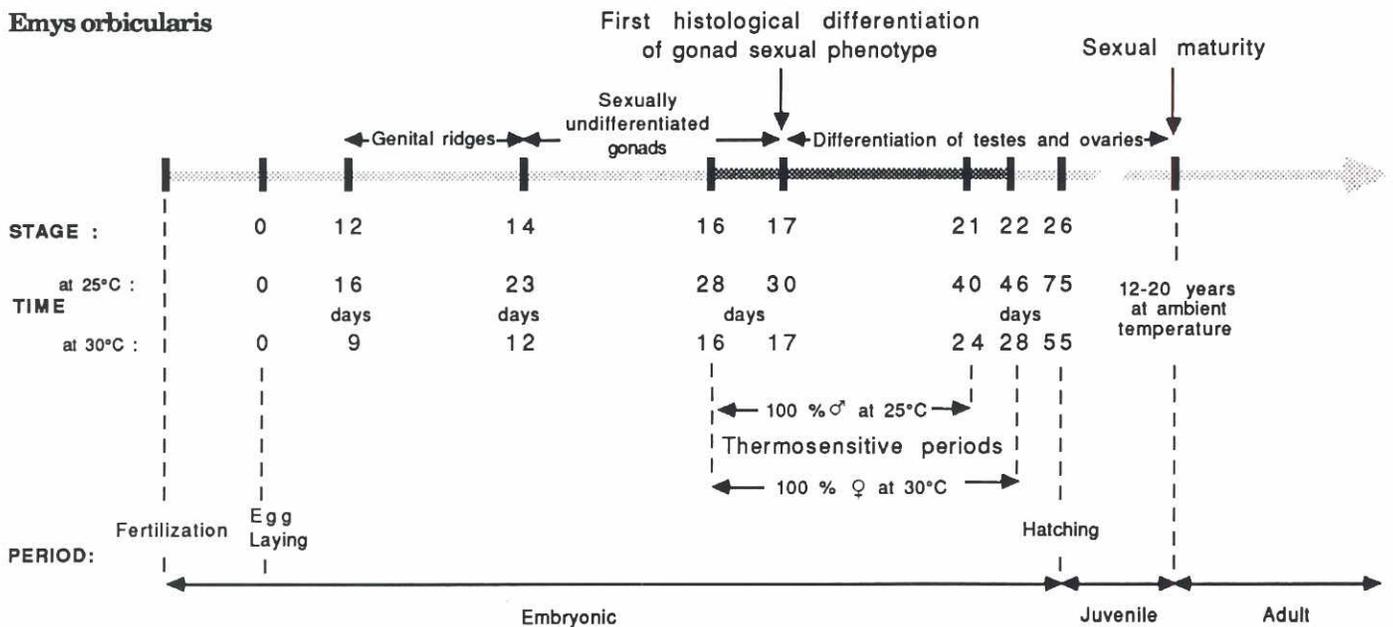


Fig. 3. Thermosensitive periods for sexual differentiation of the gonads in the salamander *Pleurodeles waltl* and the turtle *Emys orbicularis*.

Pattern 2: type Alligator mississippiensis

The response is opposite to that observed in *Emys*, with 100% phenotypic males above the critical range of temperature and 100% phenotypic females below. In *A. mississippiensis*, all the individuals issued from eggs incubated at 30°C or below were phenotypic females, whereas those issued from eggs incubated at 34°C and

above (with a high mortality at 36°C), were phenotypic males (Ferguson and Joanen, 1982, 1983). The critical range of temperature allowing both male and female differentiation is probably 31°-33°C. This pattern has been observed in 3 other species of crocodiles (*Crocodylus niloticus*, *Crocodylus siamensis*, *Caiman crocodyllus*, reviewed by Deeming and Ferguson, 1988) and seems

TABLE 4

EFFECTS OF HEAT AND COLD TREATMENTS ON SEXUAL DIFFERENTIATION IN LARVAE OF *PLEURODELES WALTL* ISSUED FROM A CROSS ZZ STANDARD MALE x ZW STANDARD FEMALE (OFFSPRING Sd-77)

Temperature	Number and percentage			Total
	Males	Intersexes	Females	
20° ± 2°C (room)	43 46.7 % (50 %)	0	49 53.3 % (50 %)	92
30°C	26 49.1 %	15 28.3 %	12 22.6 %	53
15°C	25 (44.6 %)	1 (1.8 %)	30 (53.6 %)	56

The theoretical sex ratio in conformity with genotypic sex determination is indicated in parentheses.

also to occur in two species of lizards (*Agama agama*, Charnier, 1966; *Eublepharis macularius*, Wagner, in Bull, 1980).

Pattern 3: type *Chelydra serpentina*

In this pattern, 100% phenotypic females are obtained at cool as well as at warm temperatures and 100% phenotypic males at intermediate ones, with two critical ranges in the transition intervals. In *C. serpentina*, all individuals became phenotypic females at 20° and at 30°C, whereas 100% phenotypic males were obtained at 24°C. Both sexes differentiated within the intervals 21°-23°C and 26°-29°C, with a high percentage of phenotypic males at 22° and 28°C (Yntema, 1976). Preliminary data indicate that this pattern also occurs in 9 other species of turtles (Bull, 1980; Vogt *et al.*, 1982; Gutzke and Paukstis, 1984; Ewert and Nelson, personal communication), the lizard *Gekko japonicus* (Tokunaga, 1985) and 2 species of crocodiles (*Crocodylus porosus* and *C. palustris*, reviewed by Deeming and Ferguson, 1988).

Pattern 4: type *Crocodylus johnstoni*

In *C. johnstoni*, phenotypic females were obtained at all incubation temperatures, but phenotypic males were produced only between 31° and 32.5°C with a lower percentage than that of phenotypic females (Webb and Smith, 1984).

This pattern could derive from pattern 3 by the extreme reduction of the temperature interval allowing male differentiation. It shows two threshold temperatures, one maximum and the other minimum, for male differentiation, but no threshold temperature for female differentiation. In patterns 1, 2 and 3, there are threshold temperatures for both male and female differentiation. Thus, in *Emys orbicularis* (pattern 1), the threshold temperature for male differentiation is 29.5°C while the threshold temperature for female differentiation is 28°C.

Pattern 5: type *Lacerta viridis*

Temperature does not affect sexual differentiation. Eggs of *L. viridis* were incubated either at 17.5°C, 19.5°C, 25.5° ± 0.5°C or at 35.5° ± 0.5°C. At each of these temperatures, the sex ratio was close to 1 male: 1female (Raynaud and Pieau, 1972), showing that genotypic sex determination was respected. Other species lack temperature sensitivity of sexual differentiation: the lizard *Dipso-*

saurus dorsalis (Muth and Bull, 1981), the snake *Nerodia fasciata* (Osgood, in Bull, 1980) and the turtles *Chelodina longicollis* (Georges, 1988), *Emydura signata*, *E. macquarii* (Thompson, 1988), *Clemmys insculpta* (Bull *et al.*, 1985), *Trionyx spiniferus* (Vogt and Bull, 1982) and *T. muticus* (Ewert and Nelson, personal communication).

Genotypic sex determination and temperature sex-reversal

The most irrefutable demonstration of sex-reversal under the effect of an epigenetic factor is provided by the analysis of the progeny, some of which are unisexual under conditions allowing the sexual differentiation of both sexes. Sex-linked characters and/or identification of sex chromosomes can also be looked for. All these methods were successfully applied in amphibians, whereas in reptiles only detection of a sex-linked marker was investigated.

Amphibians

Evidence of temperature sex-reversal and confirmation of the ZZ/ZW mechanism of genotypic sex determination were obtained in *Pleurodeles poireti* by crossing ZZ thermoneofemales with ZZ standard males. In the progeny reared at room temperature, only males were present (Table 5) (Dournon *et al.*, 1984). In *P. waltl*, 80 offspring (4511 juveniles and adults) from ZW thermoneomales produced at 32°C and from ZW standard females were studied for sex ratio and genetic analysis (Fig. 2). In all cases, the individuals were reared at room temperature (20° ± 2°C), and the observed sex ratios were in accordance with the theoretical expectations (Fig. 2) (Dournon and Houillon, 1983, 1984).

Peptidase-1 is a dimeric sex-linked enzyme. Its electrophoretic pattern reveals a polymorphism that has been used to identify the ZZ, ZW and WW sexual genotypes in *P. waltl* (Ferrier *et al.*, 1980, 1983; Dournon *et al.*, 1988), and the ZZ and ZW genotypes in *P. poireti* (Dournon *et al.*, 1984). For example, it was used to distinguish WW individuals from ZW ones in crosses ZW x ZW and ZW x WW of *P. waltl* (Fig. 2) (Dournon *et al.*, 1988) and to detect ZZ thermoneofemales of *P. poireti* (Dournon *et al.*, 1984).

Typing of the serologically-detectable H-Y (SD-H-Y) antigen in the non-gonadal tissues (such as blood) can also be used as a tool to identify the sexual genotype, since this antigen has been shown to characterize the heterogametic sex. In *P. waltl*, the ZW standard genotypic females are SD-H-Y positive whereas the ZZ standard genotypic males are SD-H-Y negative (Dournon and Zaborski, 1979; Zaborski, 1979). After heat treatment of the larvae, the testes of the

TABLE 5

SEX RATIO IN THE OFFSPRING ISSUED FROM A CROSS ZZ STANDARD MALE x ZZ THERMONEOFEMALE IN *PLEURODELES POIRETI*

Temperature	Number and percentage			Total
	Males	Intersexes	Females	
20° ± 2°C (room)	68 100 % (100 %)	0	0	68

The theoretical sex ratio in conformity with genotypic sex determination is indicated in parentheses.

ZW thermoneomales typed SD-H-Y negative like those of ZZ standard males, but the blood cells remained SD-H-Y positive (Zaborski, 1986).

In *P. waltl*, two characteristics of skin pigmentation are linked to the W chromosome and one is expressed in larvae, while the other is expressed in post-metamorphic individuals. As they are recessive, these characters are expressed only in WW females, which therefore are easily identifiable (Collenot *et al.*, 1989).

In *P. poireti* and *P. waltl*, heteromorphic sex chromosomes are not visible during mitosis. However, sex chromosomes are identifiable during meiosis. Indeed, differential sequences of transcription loops of lamp brush chromosomes (bivalent IV) characterize respectively the Z and the W chromosome. In a strain of *P. poireti*, the W chromosome carries a specific sequence of loops, visible under a phase contrast microscope (Lacroix, 1970). As this sequence is absent in the Z chromosome, the bivalent IV from an oocyte of a standard ZW female is heterozygotic, whereas that of an arrhenogenous ZZ female (thermoneofemale for example) is homozygotic (Lacroix, 1970; Dournon *et al.*, 1984). In *P. waltl*, differential sequences in the sex determining segments of the Z and W chromosomes appear after a heat-shock treatment or are revealed by immunostaining using monoclonal antibodies against germinal vesicles of oocytes (Lacroix *et al.*, 1985, 1990).

Reptiles

In reptiles as in amphibians, species sensitive to temperature for sexual differentiation of the gonads do not display heteromorphic sex chromosomes during mitosis, except perhaps the turtle *Staurotyphlus salvinii* (Ewert and Nelson, personal communication), which has been shown to have male karyotypic heteromorphy (Sites *et al.*, 1979). Polymorphic sex-linked enzymes such as peptidase-1 in salamanders are not known. Sexual maturity is generally reached several years after hatching, except in lizards (for example *Eublepharis macularius*, Bull, 1987). Therefore, in most species, the genetic analysis of the progeny as that performed in salamanders, cannot be reasonably undertaken. Up to now, the unique marker making possible the identification of genotypic sex is the serologically detectable H-Y antigen (SD-H-Y). Detection of this antigen was investigated in adults of 15 turtle species. In *Emys orbicularis*, SD-H-Y typing on blood and spleen cells was shown to be positive in the majority of females and negative in males (Zaborski *et al.*, 1979, 1982; Servan *et al.*, 1989), indicating a female heterogamety (ZZ/ZW sex-determining mechanism). In the opposite, in *Chelonia mydas*, blood cells were found to be SD-H-Y positive in males and negative in females, a result agreeing with an XX/XY sex-determining mechanism (Wellins, 1987). In a study performed on 14 species of turtles, Engel *et al.* (1981) found that in 13 species (including *Emys orbicularis*), the females were SD-H-Y positive and the males SD-H-Y negative, whereas in one species the opposite occurred. Therefore, both ZZ/ZW and XX/XY mechanisms of genotypic sex determination appear to exist in turtles, the ZZ/ZW being the most frequent.

The expression of SD-H-Y antigen was also carried out in young *Emys orbicularis* issued from eggs incubated at different temperatures: 25°-26°C resulting in 100% phenotypic males, 30°-30.5°C yielding 100% phenotypic females and 28.5°±0.2°C, the critical temperature allowing differentiation of both sexes. In young males from the 25°-26°C incubation, blood cells were positive in some individuals and negative in others, whereas testicular cells from all

were negative. In young females from the 30°-30.5°C incubation, blood cells again were positive in some individuals and negative in others, whereas ovarian cells were always positive (Zaborski *et al.*, 1982). These results showed that in gonads, the SD-H-Y expression is closely associated with ovarian structure, as it is in *Pleurodeles waltl* (Zaborski, 1986) and other vertebrates presenting a ZZ/ZW sex-determining mechanism. In blood, however, SD-H-Y expression does not follow the sexual phenotype but probably remains in conformity with the sexual genotype, the SD-H-Y positive individuals from both incubation temperatures being genotypic females and the SD-H-Y negative ones being genotypic males. Thus, the phenotypic females which were blood SD-H-Y negative and the phenotypic males which were blood SD-H-Y positive were considered to be sex-reversed (Zaborski *et al.*, 1982). From this interpretation, one could expect that in the critical range of temperature, the sexual phenotype would be in accordance with the sexual genotype. The results obtained in young individuals from the 28.5 ± 0.2°C incubation agree with this hypothesis: in all phenotypic males, blood cells typed SD-H-Y negative, whereas in most phenotypic females, they typed SD-H-Y positive (Zaborski *et al.*, 1988). From all these results, we can postulate that, in *Emys orbicularis*, sexual phenotype generally conforms with sexual genotype (ZZ or ZW) within the critical range of temperature, whereas above and below this range, the effects of temperature override genotypic sex determination.

As shown by studies of the expression of SD-H-Y antigen in adults of other turtle species, GSD probably exists in most if not all species whose gonadal differentiation is influenced by temperature. Therefore, the distinction between two different mechanisms of sex determination in reptiles – genotypic sex determination (GSD) and temperature-dependent sex determination (TSD) (Bull, 1980) – although usual, does not correspond to all experimental data and must be reconsidered (Raynaud and Pieau, 1985).

Temperature-sensitive stages for sexual differentiation of the gonads

Determination of the temperature-sensitive stages (or periods) for sexual differentiation of the gonads has been investigated in the salamander *Pleurodeles waltl* (Dournon and Houillon, 1985), and in several species of reptiles including lizards (Bull, 1987), turtles (Yntema, 1979; Bull and Vogt, 1981; Pieau and Dorizzi, 1981; Yntema and Mrosovsky, 1982) and crocodylians (Ferguson and Joanen, 1983; Webb *et al.*, 1987; Deeming and Ferguson, 1988; Lang *et al.*, 1989). The effects on the sex ratio of temperature changes at different larval or embryonic stages were studied.

In *Pleurodeles waltl*, larvae were first reared at room temperature (20° ± 2°C) up to a given stage, then shifted to 30°, 31° or 32°C (male-producing temperatures) for a definite period and finally returned to room temperature. Control larvae were reared at room temperature. The study was performed on a total of 2304 larvae from 15 different mating pairs. It was shown that the thermosensitivity of sexual differentiation of the gonads differed between individuals but did not differ significantly between the mating pairs. Moreover, 31° and 32°C were more efficient temperatures in reversing the female phenotype than 30°C. To obtain sex-reversal at 30°C, the genotypic females had to be treated between stages 43 and 45 (Gallien and Durocher, 1957), but even then not all responded, no matter how long they were exposed to 30°C after stage 45. At 31° and 32°C, 100% of genotypic females were sex-

reversed after a minimal period of exposure between stage 43 and 54 (Fig. 3), the stages 43 to 45 again being critical for the efficiency of the treatment (Dournon and Houillon, 1985).

In reptiles, eggs were shifted at different stages of embryonic development from a male-producing temperature to a female-producing temperature (or vice versa) for the remainder of development, or were submitted to pulses of one incubation temperature on the background of a second temperature. The temperature-sensitive stages were defined as those comprised between the two stages before and after which a given temperature (male- or female-producing) had no effect on the sex ratio (Bull, 1980). This interval has been shown to be different for male and female differentiation and to vary according to the temperature chosen. For example, in *Emys orbicularis*, 35°C is more feminizing than 30°C and the thermosensitive period for female differentiation is shorter at 35°C than at 30°C, although the rate of embryonic development is nearly the same at both temperatures. Therefore, the proposal has been made to define the thermosensitive periods as the minimal duration of exposure at a male- or a female-producing temperature which results either in 100% phenotypic males or in 100% phenotypic females (Pieau and Dorizzi, 1981; Pieau, 1982). Thus, in *Emys orbicularis* the thermosensitive periods extend respectively, from stage 16 to stage 21 for male differentiation at 25°C, and from stage 16 to stage 22 for female differentiation at 30°C (Fig. 3). These periods last 11-12 days at 25° and 30°C and correspond to the first steps of histological differentiation of the gonads (Pieau and Dorizzi, 1981). Similar results have been generally obtained in other reptilian species. In *Pleurodeles waltl*, the gonads appear to be histologically undifferentiated for the greater part of the thermosensitive period for sexual inversion of genotypic females (compare with *Emys orbicularis* in Fig. 3).

Altogether, the results of shift experiments from one temperature to another in amphibians and in reptiles suggest a dose-effect of temperature, with production of a substance (or substances) either feminizing or masculinizing above a certain threshold, and not below. Steroid hormones are good candidates for such actions.

Involvement of steroid hormones in sexual differentiation of the gonads

Functional inversion of sexual phenotype under the effects of sex steroids was demonstrated in several species of amphibians (reviewed by Gallien, 1959, 1962). In *Pleurodeles waltl*, as in other species displaying a ZZ/ZW sex-determining mechanism, the estrogenic treatment of larvae led to partial or complete sex-reversal of ZZ genotypic males. At room temperature, the progeny of ZZ neofemales crossed with ZZ standard males were unisexual (100% males) as expected (Gallien, 1951, 1954). Moreover, in this species, the masculinizing effects of high temperatures on genotypic female larvae were counteracted by estrogen treatment (Zaborski, 1986). Feminizing effects of estrogens on sexual differentiation of the gonads were also obtained in reptiles (reviewed by Raynaud and Pieau, 1985). In *Lacerta viridis*, a species with a ZZ/ZW sex-determining mechanism like *P. waltl*, injection of estradiol benzoate into eggs prior to the first signs of histological differentiation of the gonads led to inhibition (partial or complete according to the dose of injected hormone) of development of testicular cords (or tubes) and formation of a thin ovarian-like cortex on the lateral sides of the gonads. This cortex was stimulated when gonadotropin was asso-

ciated with estradiol (Raynaud, 1967). Similar results were subsequently obtained in *Testudo graeca* and *Emys orbicularis* after injection of estradiol benzoate into eggs incubated at male-producing temperatures during the thermosensitive period for male differentiation (Pieau, 1970, 1974). More recently, the feminizing effects of estradiol were extended to other turtle species (3 with temperature sensitivity of gonadal differentiation, 1 without), the alligator *Alligator mississippiensis* and the lizard *Eublepharis macularius* (Gutzke and Bull, 1986; Bull *et al.*, 1988; Crews *et al.*, 1989). The sensitive period for hormonal effects on gonadal development apparently coincides with the period of normal gonadal differentiation (Gutzke and Chymiy, 1988).

All these data suggest that estrogens are involved in differentiation of the gonads as a function of temperature. Above a threshold level of estrogen gonads would differentiate into ovaries, whereas below it gonads would differentiate into testes. In this view, estrogen content would be superior to the threshold level at feminizing temperatures and inferior to this level at masculinizing ones. Preliminary (unpublished) data in *Emys orbicularis* indicate that the level of estrogens (estradiol-17 β and estrone) into the gonads is higher at 30°C (feminizing temperature) than at 25°C (masculinizing temperature). Moreover, injection of an anti estrogen (tamoxifen) into eggs incubated at 30°C, before or at the beginning of the thermosensitive period for female differentiation, resulted in the differentiation of testicular tubes (or cords) in the the gonads (Dorizzi *et al.*, 1986-1987 and paper in preparation). Similar although not so obvious results were obtained with aromatase inhibitors (unpublished results).

In *Chelydra serpentina*, administration of estradiol antiserum into eggs incubated at female-producing temperature resulted in some individuals having ambiguous gonads (Crews *et al.*, 1989). All these data suggest that in thermosensitive species, the gonadal production of estrogens is temperature-dependent. A thermosensitive factor could therefore be implicated in the regulation of synthesis of the cytochrome-P 450 aromatase, the enzyme responsible for aromatization of androstenedione into estrone and of testosterone into estradiol-17 β (Pieau *et al.*, 1987).

Concluding remarks

Temperature has been shown to influence sexual differentiation of the gonads in many species of amphibians and reptiles. As a general rule, in amphibians, genotypic sex determination is respected in a wide range of temperature and temperature sex-reversal probably rarely occurs in nature. However, YY males were observed in natural populations of Japanese anurans (Kawamura and Nishioka, 1977), indicating the possible influence of temperature or of some other environmental factor.

In reptiles, both sexes often differentiate only within a narrow range of temperature and temperature sex-reversal in natural conditions is probably more frequent than in amphibians. However, in many species, such as *Emys orbicularis*, eggs are deposited at shallow depths and are submitted to external variations of temperature (nycthemeral rhythm, weather changes), with maxima above the critical range of temperatures and minima below this range (Pieau, 1982). These fluctuations of temperature allow both males and females to differentiate, and the proportion of sex-reversed individuals in natural populations appears to be relatively low (Servan *et al.*, 1989).

The problem of adaptive value of temperature sensitivity of sexual differentiation in reptiles has been addressed by many authors (see for example Bull, 1980, 1983; Ferguson and Joanen, 1982, 1983; Bull and Charnov, 1989; Deeming and Ferguson, 1989) on the basis of the general model of Charnov and Bull (1977) who examined conditions favoring this phenomenon. Although this "model is not yet supported by the data, it is also not evidently inconsistent with the data either" (Bull and Charnov, 1989) and the question remains open.

As a general rule, temperature sensitivity of sexual differentiation in amphibians and reptiles is linked with the absence of heteromorphism or a slight degree of differentiation of sex chromosomes. Since heteromorphism of these chromosomes appears to be of relatively recent origin (Bull, 1980), it can be assumed that this phenomenon is a much more primitive characteristic and might represent conservation of a temperature-dependent regulatory mechanism existing in ancestral forms (Pieau *et al.*, 1989).

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