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Temperature-dependent sex determination and gonadal differentiation in reptiles

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Abstract. In many reptile species, sexual differentiation of gonads is sensitive to temperature during a critical period of embryonic development (thermosensitive period, TSP). Experiments carried out with different models among which turtles, crocodilians and lizards have demonstrated the implication of estrogens and the key role played by aromatase (the enzyme complex that converts androgens to estrogens) in ovary differentiation during TSP and in maintenance of the ovarian structure after TSP. In some of these experiments, the occurrence of various degrees of gonadal intersexuality is related to weak differences in aromatase activity, suggesting subtle regulations of the aromatase gene at the transcription level. Temperature could intervene in these regulations. Present studies deal with cloning (complementary DNAs) and expression (messenger RNAs) of genes that have been shown, or are expected, to be involved in gonadal formation and/or differentiation in mammals. Preliminary results indicate that homologues of *AMH*, *DAX1*, *SF1*, *SOX9* and *WT1* genes with the same function(s) as in mammals exist in reptiles. How these genes could interact with aromatase is being examined.

Key words. Gonadal differentiation; intersexuality; temperature; estrogens; aromatase; *AMH*, *DAX1*, *SF1*, *SOX9*, *WT1* genes; reptiles.

Introduction

As fish and amphibians, reptiles exhibit different mechanisms of sex determination. In some species including snakes, many lizards and a minority of turtles, sex is determined by a gene or genes carried on sex chromosomes, according to male (XY/XX) or female (ZW/ZZ) heterogamety. The ZW/ZZ mechanism only exists in snakes, whereas both XY/XX and ZW/ZZ mechanisms are found in lizards and turtles. In this genotypic (GSD, [1, 2]), also called chromosomal sex determination (CSD, [3]), sex chromosomes vary from strongly heteromorphic (as in Viperidae) to slightly or not heteromorphic (as in Boidae and turtles). In other reptiles, all oviparous, sexual differentiation of gonads is sensitive to the incubation temperature of the eggs during a critical period of embryonic development.

In 1966, biased sex ratios related to different conditions of egg incubation (substrate, temperature) were reported in a lizard, *Agama agama*, suggesting an influence of temperature [4].

In 1971–72, gonadal differentiation was shown to be temperature-sensitive in the European freshwater turtle *Emys orbicularis* and the Mediterranean tortoise *Testudo graeca* [5, 6]. Later, the study of the effects of a wide range of temperatures demonstrated the influence of egg incubation temperature on the hatchling sex ratio also in an American turtle, *Chelydra serpentina* [7]. As of the end of the 1970s, these pioneer works were extended to many species and showed that the so-called temperature-dependent sex determination (TSD, [1, 2]) is widespread in reptiles. Thus TSD has been found in all crocodilians studied so far (i.e. half of the extant species), most turtles, some lizards [8] and more recently in the two living closely related species of *Sphenodon* [9].

In TSD reptiles, masculinizing temperatures yield 100% or a majority of males, whereas feminizing temperatures yield 100% or a majority of females. In the transition range(s) of temperature (TRT), males and females and sometimes intersexes are obtained. However, the responses to incubations at different constant tempera-

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tures vary according to three different patterns. In many turtles including all sea turtles, temperatures below TRT are masculinizing, whereas those above TRT are feminizing. In *Sphenodon* and some lizards, the inverse has been described. In other species of turtles and lizards, and in crocodilians, two TRTs have been found: intermediate temperatures are masculinizing, whereas lower and higher temperatures are feminizing. Possibly, testing a larger range of temperatures would reveal that species classified in the second pattern actually belong to the third pattern [8].

Within TRT, the pivotal temperature has been operationally defined as that temperature which, in a given species, a population or a clutch, yields 50% males and 50% females [10].

The thermosensitive period (TSP) for gonadal differentiation has been determined in a few species of crocodilians and turtles and in a lizard. For this purpose, shifts from a male- to a female-producing temperature and vice versa were performed at different embryonic stages, and sex ratio was examined at the time of hatching. The duration of TSP represents 18–30% of embryonic development, since the developmental stage of embryos at oviposition is variable. However, in all cases, TSP corresponds to the first stages of gonadal differentiation as revealed by classical histology [8].

The different patterns of TSD and their features (pivotal temperature, transitional range of temperature, thermosensitive period) have been established from incubations at constant temperatures in the laboratory. In nature, nest temperature fluctuates, in particular in shallow nests, which are submitted to nycthemeral rhythm and weather changes [11]. In such conditions, the hatchling sex ratio depends on the proportion of embryonic development that occurs above and below the pivotal temperature during TSP [11, 12].

The reader will find in several previous reviews information on the different patterns of TSD, their features and their ecological and evolutionary implications [1, 2, 13–17]. Other reviews deal mainly with endocrinological aspects of TSD, in particular the role of steroids and steroidogenic enzymes in gonadal sex differentiation [8, 18–23]. Several hypotheses on the molecular mechanisms of TSD have already been formulated [8, 18, 23–27], although molecular approaches are only beginning to be used in reptiles.

In this review, we have selected cellular, molecular and physiological data obtained at the gonadal level only, i.e. data that, in our opinion, are fundamental to elucidate the mechanism of TSD in reptiles and to allow fruitful comparisons with other vertebrates.

Gonadal differentiation and growth as a function of temperature

Gonadal differentiation has been described in several reptiles, including both GSD and TSD species [28]. Some histological descriptions were made long before the discovery of TSD, in species which are now known to exhibit TSD, such as the turtles, *Chrysemys picta* [29] and *Sternotherus odoratus* [30], and the alligator *Alligator mississippiensis* [31]. Recent studies were performed in other TSD turtles, including *Testudo graeca* [32, 33], *Emys orbicularis* [34], *Dermochelys coriacea* [35], *Lepidochelys olivacea* [36, 37] and *Trachemys scripta* [38, 39], and again in the alligator *A. mississippiensis* [24, 40–42]. In the latter, histological studies, immunohistological localization of two stuctural proteins (laminin and cytokeratin) and ultrastructural studies were carried out [41–43].

Gonadal growth at male- and female-producing temperatures was analyzed in *Emys orbicularis* from the beginning of TSP to hatching by determining gonadal protein content [44].

The main morphological changes occurring during gonadal differentiation are similar in all turtle species and in the alligator, although they display some specific characteristics. Figure 1 schematizes these changes from the formation of the gonadal primordium to the structure of the gonads at hatching. It is based mainly on data obtained in *E. orbicularis* and *A. mississippiensis* [34, 41–44].

As in other vertebrates, the gonadal primordia ('genital ridges') develop as thickenings of the coelomic epithelium on the ventromedial surface of the mesonephric kidneys, on each side of the dorsal mesentery. Primordial germ cells are scattered in this epithelium ('germinal epithelium') and, less frequently, in the underlying mesonephric mesenchyme. In turtles, germ cells are in the 'posterior germinal crescent' at the end of gastrulation [30, 45, 46]; they then migrate through the dorsal mesentery to reach the gonad anlagen.

During development of genital ridges, epithelial cells are added to the initial mesenchyme in the inner part of the gonads (medulla). These epithelial cells proliferate from different places: the external epithelium of Bowman's capsule of Malpighian corpuscles, the coelomic epithelium bordering mesonephric kidneys on the lateral side of each gonad (at the place of previous nephrostomes) and the germinal epithelium itself.

Cells that proliferate from both the Malpighian corpuscles and the lateral coelomic epithelium are generally small, darkly staining and are organized into thin cords in the dorsal part of the gonadal primordium. These cords are the anlagen of the rete cords.

Cells that proliferate from the germinal epithelium also give thin cords of epithelial cells which penetrate into the underlying initial mesenchyme. These 'medullary cords' or 'sex cords' [29] become rapidly outlined with a basement membrane. In *A. mississippiensis*, sex cords are composed of numerous small, irregularly shaped cells, and occasionally of some enlarged somatic cells which have been considered but not determined to be pre-Sertoli cells. The continuity of the basement membrane between the germinal epithelium and the sex cords has been demonstrated by positive laminin immunoreactivity. Cytokeratin is present in the germinal epithelium and in some scattered cells of the medulla (pre-Sertoli cells?). Sex cords and rete cords enter into contact in the inner part of the gonad; thus a mixture of cells from these two types of cords is possible there.

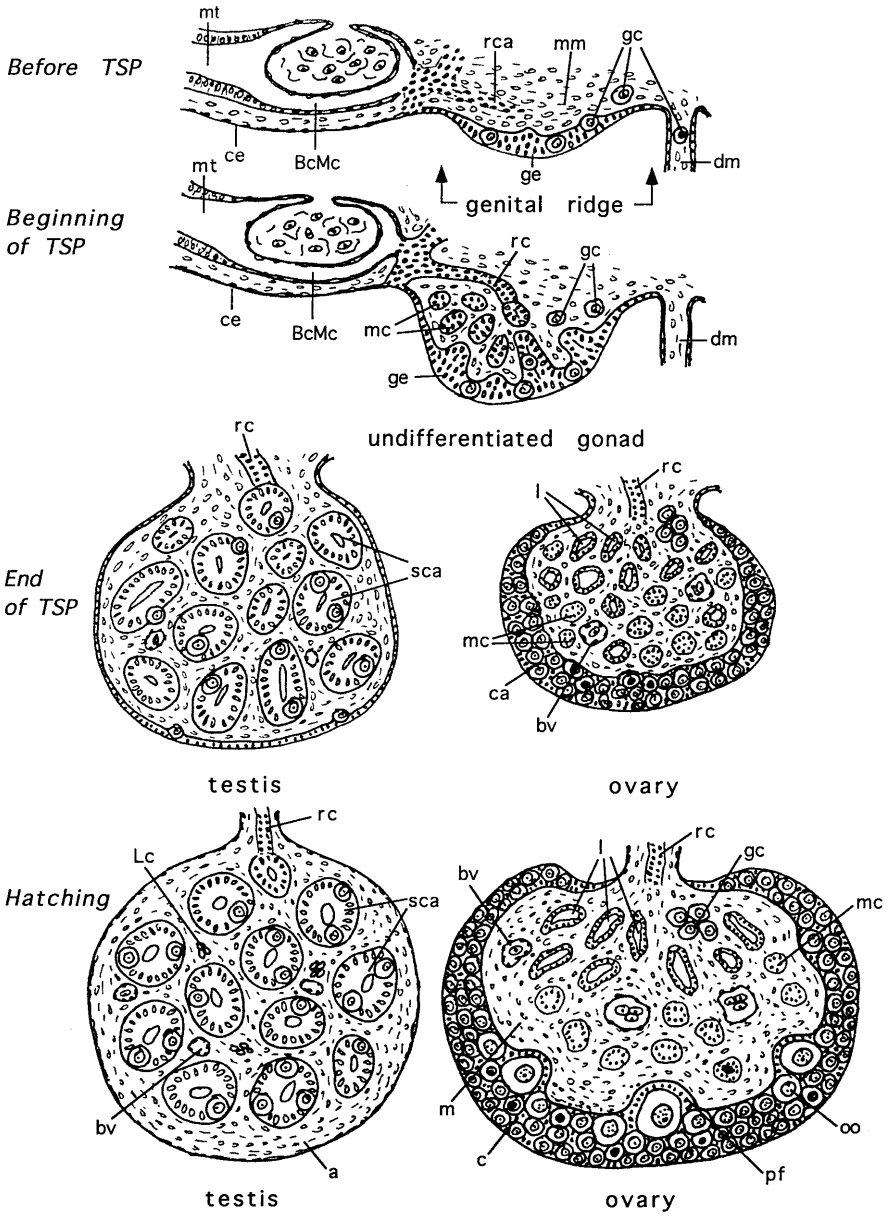

Figure 1. Gonadal differentiation in reptiles with temperature-dependent sex determination: a, albuginea; BcMc, Bowman's capsule of Malpighian corpuscle; bv, blood vessel; c, cortex; ca, cortex anlage; ce, coelomic epithelium; dm, dorsal mesentery; gc, germ cells; ge, germinal epithelium; l, lacunae; Lc, Leydig cells; m, medulla; mc, medullary cords; mm, mesonephric mesenchyme; mt, mesonephric tube; oo, oocyte; pf, primordial follicle; rc, rete cord; rca, rete cord anlage; sca, seminiferous cord anlagen.

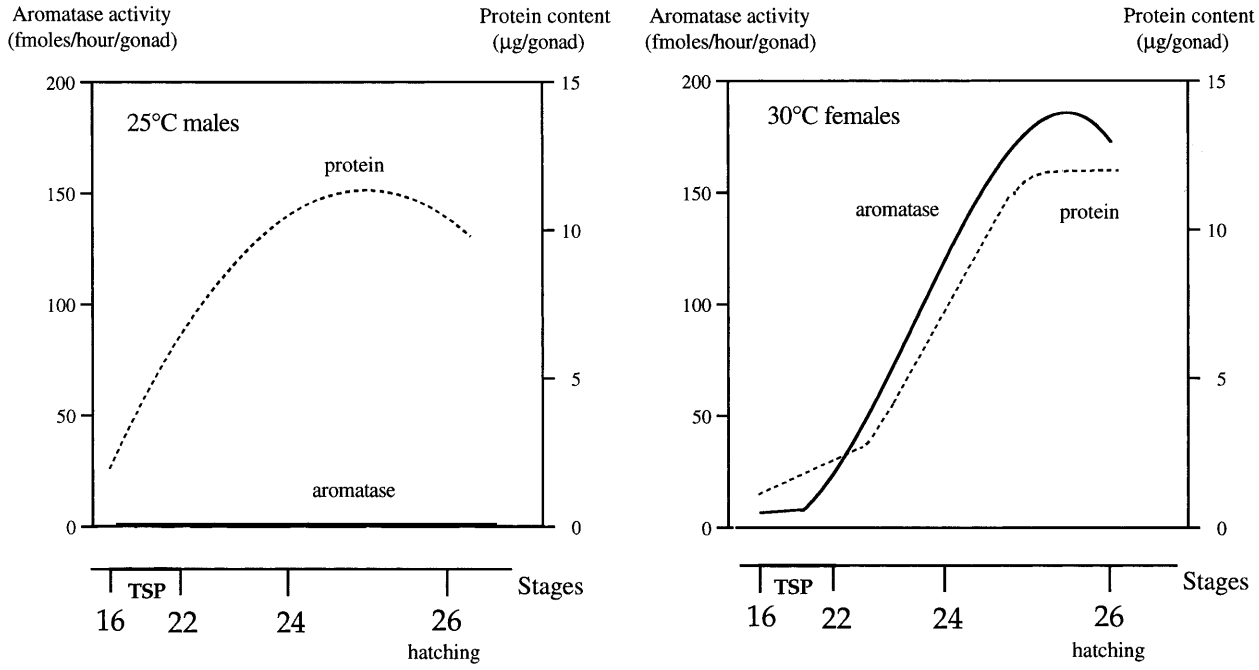

Figure 2. Gonadal aromatase activity and gonadal protein content from the beginning of the thermosensitive period (TSP) to hatching in embryos of *E. orbicularis* incubated at 25 °C (male-producing temperature) and at 30 °C (female-producing temperature). At 25 °C, aromatase activity is very low; protein content increases regularly during and after TSP and somewhat decreases at hatching following the development of testicular cords (future seminiferous cords). At 30 °C, aromatase activity increases exponentially during and after TSP and somewhat decreases at hatching; protein content increases weakly during TSP and strongly after TSP, following the development of ovarian cortex. The curves of aromatase activity and protein content are almost parallel (modified from [44]).

This gonadal structure is observed in both sexes up to the very beginning of TSP. Thus, gonads are usually considered to be undifferentiated at this time; however, sex cords are slightly thinner at female- than at male-producing temperatures. Gonadal sex differentiation occurs during TSP.

In differentiating testes, the germinal epithelium flattens and remains cytokeratin-positive. Germ cells leave it and migrate between the epithelial cells of the medullary cords. In these cords, more and more cells acquire the Sertolian characteristics. Cytokeratin become concentrated in the basal cytoplasm of Sertolian cells. Medullary cords thus form the seminiferous cord anlagen with a rounded contour well delineated by a laminin-immunoreactive basement membrane. During TSP, testis growth is regular due to the development of seminiferous cords in the medulla (fig. 2).

In differentiating ovaries, the germinal epithelium thickens due to the in situ proliferation of epithelial cells and germ cells which give rise to oogonial nests. Thus, an ovarian cortex develops. By the end of TSP, some germ cells have generally entered into meiosis. Strong cytokeratin immunoreactivity is observed in epithelial cells of the cortex, but germ cells are cytokeratin-negative. The cortex is separated from the medulla by a laminin-positive basement membrane. In the medulla, the putative pre-Sertoli cells never become as numerous as in differentiating testes and do not complete differentiation into Sertoli cells. A laminine-positive basement membrane outlines the medullary cords. However, these cords become thin and appear fragmented, being separated by dark interstitial cells and capillary blood vessels. Another difference with seminiferous cords is that in ovarian medullary cords, cytokeratin is localized in the apical cytoplasm of epithelial cells, instead of the basal part in Sertolian cells. In both testes and ovaries, thin rete cords remain localized in the dorsal part of the medulla.

Fragmentation of ovarian medullary cords has often been interpreted as a regression of these cords [36, 37, 39, 41–43]. However medullary cords, or at least some of them, do not completely regress but evolve as small lacunae bordered by a flat epithelium, as clearly shown in the turtles *Testudo graeca* [33] and *D. coriacea* [35].

During TSP, ovary growth is less important than testis growth. This differential growth can easily be explained by the fact that the ovarian medulla is strongly reduced compared with the testis medulla and that the ovarian

cortex has a weak development (fig. 2). After TSP, a thin albuginea, composed of a few layers of fibroblasts, differentiates and surrounds the testes. At the end of the embryonic development, the diameter of testicular cords decreases somewhat, and consequently the protein content of the testis also decreases (fig. 2). Leydig cells begin to differentiate in interstitial tissue. Apoptotic cell death is observed in differentiating albuginea and in sparse cells of seminiferous cords (N. Richard-Mercier, M. Dorizzi and C. Pieau, unpublished results). In ovaries, proliferation of germ cells and the entry of these cells into meiosis continue in the cortex which thus strongly thickens. Ovarian protein content also strongly increases and by the end of the embryonic development, it is similar or even surpasses that of testes (fig. 2).

At hatching, primordial follicles containing growing oocytes are generally observed in the internal part of the ovarian cortex [34]. However in some species, such as the marine turtles *D. coriacea* [35] and *L. olivacea* [36], germ cells do not enter into meiosis during embryonic development.

These studies of gonadal differentiation in TSD reptiles show that both testicular seminiferous cords and ovarian medullary cords/lacunae derive from proliferation of the surface epithelium (germinal epithelium) of the gonads. Cells that proliferate from the Bowman's capsule of Malpighian corpuscles and the coelomic epithelium on the lateral side of the gonadal primordium give rise to rete cords and could contribute to a limited extent to the edification of testicular and ovarian medullary cords. Follicle cells (future granulosa cells) also appear to derive from the germinal epithelium, whereas interstitial cells between testicular and ovarian medullary cords/lacunae appear to derive from the initial mesonephric mesenchyme underlying the germinal epithelium.

Involvement of steroids in gonadal differentiation

At the beginning of the 1970s, when TSD was discovered in turtles, it was well known that embryonic or larval treatments with synthetic estrogens could feminize genetic male individuals up to sex-reversal in birds, reptiles (lizards), amphibians and fish. The development of ovotestes had been obtained in marsupials after early estrogenic treatment of young genetic males in the pouch. However, treatment of gravid females with estrogens or androgens had not modified the sexual differentiation of gonads of the fetuses in eutherian mammals. Androgenic treatments had led to sex reversal in some fish and amphibian species but had yielded paradoxical results in other species and in reptiles and birds [18].

In this context, the effects of injection of androgen (testosterone propionate) into the eggs were studied in *E. orbicularis* [34] and those of estrogen (estradiol benzoate) were examined in *E. orbicularis* [34] and *T. graeca* [32]. Testosterone did not reverse gonadal sexual phenotype at a female-producing temperature but induced formation of an ovarian-like cortex at the surface of the testes at a male-producing temperature. Estradiol induced various degrees of gonadal feminization at a male-producing temperature, from ovotestis to ovary, depending on the embryonic stage at the time of injection and on the dose of injected steroid. The structural similarity between estradiol-induced and temperature-induced ovaries was striking. Likewise, hormonal-induced ovotestes resembled ovotestes obtained in some individuals at the pivotal temperature in *E. orbicularis* [28, 47], indicating a relationship between gonadal structure and estrogen levels.

In another approach, histochemical detection of 3β-hydroxysteroid dehydrogenase-5-ene-4-ene isomerase (3β HSD), a key enzyme in steroid biosynthesis, was carried out, on the same sections, in the adrenal cortex and gonads of *E. orbicularis* and *T. graeca* embryos, using dehydroepiandrosterone (DHA) as a substrate. Before TSP, 3β HSD activity was already strong in the adrenal cortex, and a lower but significant level of enzyme activity was also present in the medullary epithelial cords of the undifferentiated gonad. However, in gonads, activity was higher at masculinizing temperature than at feminizing temperature, whereas in adrenal cortex, activity was similar at both temperatures. During gonadal differentiation (corresponding to TSP), 3β HSD activity increased in testicular cords at a male-producing temperature, whereas it decreased and disappeared in the ovarian medulla at a female-producing temperature. In the adrenal cortex, 3β HSD activity became very strong at both temperatures [34]. These results indicated that steroidogenesis is present very early in both adrenal cortex and gonad anlagen and that in the gonads, but not in the adrenal cortex, synthesis of steroidogenic enzymes or synthesis of some of them is influenced by the incubation temperature.

Based on these preliminary data, two approaches were conducted in *E. orbicularis* and other TSD reptilian species: (i) the comparison of steroid pathways in the gonads of embryos incubated at different temperatures, and (ii) the study of the involvement and role of estrogens and androgens in gonadal sex differentiation.

Evidence of the early presence of active steroidogenic enzymes and temperature-dependent steroidogenesis in the gonads

A preliminary study performed in *E. orbicularis* embryos after TSP had shown that the level of endogenous

gonadal steroids was very low and difficult to quantify with the radioimmunoassay techniques used [48]. A sensitive technique, combining two successive chromatographies (HPLC and TLC) and autoradiography was thus developed, allowing both visualization and quantification of the metabolites synthesized by gonads incubated with steroid precursors [49]. In *E. orbicularis*, it is possible to separate gonads from the adrenal-mesonephric complexes as early as the beginning of TSP. Pools of gonads from embryos during or after TSP, and incubated at 25 °C (males) or at 30 °C (females), were incubated with tritiated pregnenolone, progesterone, dehydroepiandrosterone or androstenedione as substrates. At these different stages, the gonads of *E. orbicularis* were able to metabolize these precursors, showing the presence of active steroidogenic enzymes. A general scheme of steroidogenesis in the gonads was thus deduced from this study (fig. 3).

Among steroidogenic enzymes, 5α-reductase and 20β-hydroxysteroid oxidoreductase had the highest activity at both temperatures. The 3β HSD activity was significantly higher in differentiating testes at 25 °C than in differentiating ovaries at 30 °C, thus confirming previous observations made with a histochemical method [34].

Using androstenedione as a substrate, only trace amounts of estrogens were detected on autoradiograms. Therefore, another technique (the tritiated water assay) was used to measure the activity of aromatase, the enzyme complex that converts androstenedione to estrone, and testosterone to estradiol-17β (fig. 3). In the aromatase assay, temperature had no significant effect on enzyme activity. Therefore, the differences observed in gonadal aromatase activity are due to differences in the amount of the enzyme in the gonads. In gonads of embryos incubated at 25 °C, aromatase activity remained very low from the beginning of TSP up to hatching. In gonads of embryos incubated at 30 °C, aromatase activity was also very low at the beginning of TSP, but increased exponentially during TSP and peaked after TSP; there was a slight decrease of activity around hatching [50]. It is interesting to note that in ovaries, aromatase and growth curves are almost parallel (fig. 2).

The effects of temperature shifts on gonadal aromatase activity and gonadal structure were subsequently studied. In embryos of *E. orbicularis* shifted from 25 to 35 °C (a highly feminizing temperature, [51]) during TSP and then exposed for 1 to 8 days to 35 °C, the increase in gonadal aromatase activity was exponential reflecting amplification of aromatase synthesis corresponding to the feminization of the gonad [52].

From these data in *E. orbicularis*, it appears that synthesis (and thus the activity) of at least two gonadal

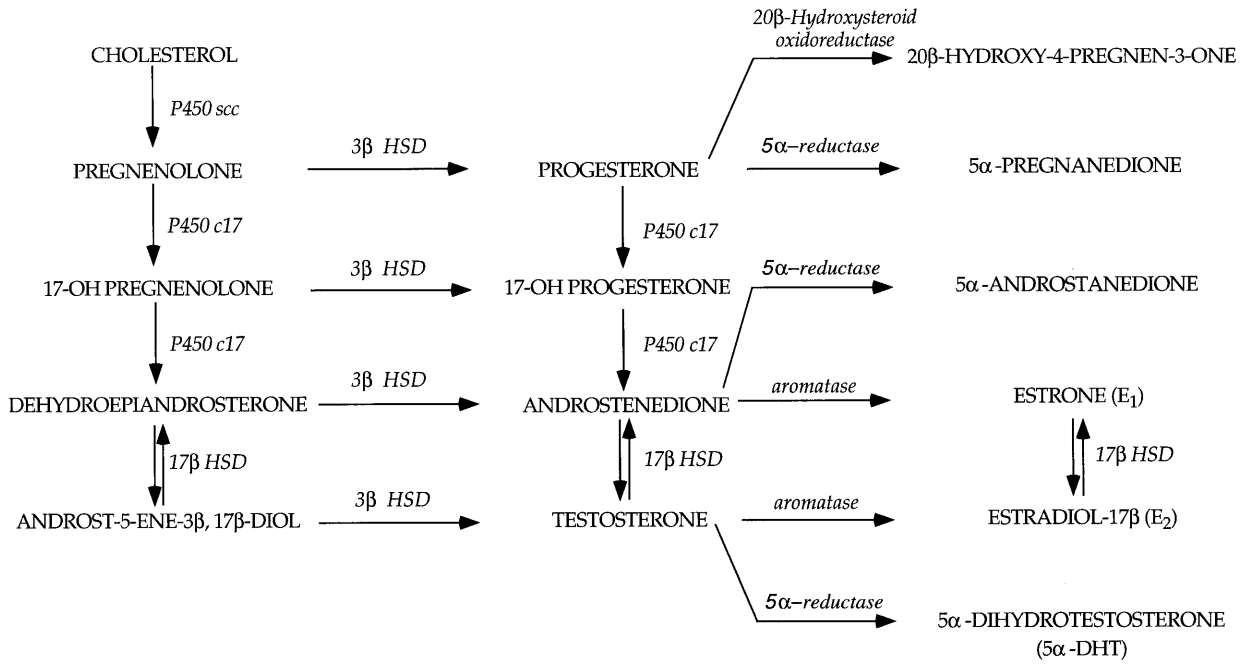

Figure 3. Major steroid pathways in the gonads of *E. orbicularis* embryos. P450scc, cholesterol side-chain cleavage cytochrome P450; P450c17, cytochrome P450 catalyzing both 17α-hydrolase and 17-20-lyase activities; 3β HSD, 3β-hydroxysteroid dehydrogenase-5-ene-4-ene isomerase; 17β HSD, 17β-hydroxysteroid dehydrogenase (modified from [8]).

steroidogenic enzymes, 3β HSD and aromatase, is different at male- and female-producing temperatures. Similar results were obtained in some other TSD species. Thus, a higher 3β HSD activity in testes than in ovaries was also shown by steroid metabolism studies in the marine turtle *D. coriacea* (G. Desvasges and C. Pieau, unpublished results), and by histochemical detection of the enzyme in *T. graeca* [6] and *A. mississippiensis* [40]. The role of 3β HSD in gonadal sex differentiation remains to be investigated.

Aromatase activity was measured with the tritiated water assay in *D. coriacea* and two crocodilians, *Crocodylus porosus* and *A. mississippiensis*. In *D. coriacea*, the gonadal aromatase activity profiles corresponding to male- and female-producing temperatures, were very similar to those in *E. orbicularis*. Again, shifts from a male- to a female-producing temperature resulted in an increase of aromatase activity only during the thermosensitive period for gonadal differentiation [53].

In crocodilians, the gonads were not separated from the adrenal-mesonephric complexes before hatching. Thus, in embryos, aromatase activity was measured in the gonad-adrenal-mesonephros (GAM) complexes. Activity remained low in the male complex, whereas it increased in the female complex during ovary differentiation. However, differences were found only after TSP: at hatching, high levels of aromatase activity were detected in the ovary of females, whereas in males they were very low and similar in both the testis and the adrenal-mesonephros [54, 55]. Problably, as in *E. orbicularis*, differences in aromatase activity existed between testis and ovary at earlier stages of development, but they could be masked by the aromatase activity of the adrenal-mesonephros part of the GAM. Results suggest that in crocodilians as in turtles, temperature directly or indirectly influences aromatase synthesis in the gonads during embryonic development [55].

Experimental evidence of the involvement of estrogens in ovary differentiation

During TSP, high levels of estrogen content were correlated with high aromatase activities in the gonads of the turtle *D. coriacea* and *E. orbicularis* [53, 56]. Moreover, a variety of experiments performed in different TSD species provided evidence for estrogen involvement in ovary differentiation. They consisted in examining the effects of early treatments (before and/or during TSP) with estrogens, antiestrogens or aromatase inhibitors on gonadal differentiation at male- and female-producing temperatures. Estrogens are normally synthesized in the gonads and also in the brain and adipose tissue; antiestrogens compete with estrogens at the level of estrogen receptors and aromatase inhibitors prevent the conversion of androgens to estrogens (see fig. 3). Among several antiestrogens and aromatase inhibitors used, tamoxifen and two nonsteroidal aromatase inhibitors, fadrozole (CGS 16949 A) and letrozole (CGS 20267) provided the more convincing results.

Table 1. Effects of early treatments (before and/or during the thermosensitive period) with estrogens (estradiol benzoate or estrone), an antiestrogen (tamoxifen), or nonsteroidal aromatase inhibitors (fadrozole or letrozole) on gonadal differentiation in *E. orbicularis*.

Incubation temperature	Treatment	Gonadal structure
25 °C (Male-producing)	control	testis
	estradiol benzoate or estrone	ovary
	tamoxifen	ovotestis
	tamoxifen + estradiol benzoate	ovotestis
	fadrozole or letrozole	testis
30 °C (Female-producing)	control	ovary
	estradiol benzoate	ovary
	tamoxifen	ovotestis
	tamoxifen + estradiol benzoate	slightly masculinized ovaries
	fadrozole or letrozole	ovotestis or testis

Let us examine the results obtained in *E. orbicularis* where a complete experimental series was performed (table 1).

In embryos incubated at 25 °C (male-producing temperature), exogenous estrogens (estradiol benzoate or estrone) induced ovarian differentiation. The antiestrogen tamoxifen had previously been reported to have both agonistic and antagonistic effects on mammalian tissues. Both effects were obtained at 25 °C in *E. orbicularis*. Tamoxifen induced the formation of an ovarian-like cortex at the surface of the testes which thus became ovotestes (agonistic effect). When tamoxifen was applied simultaneously with estradiol benzoate, the gonads were also ovotestes, showing that tamoxifen had prevented the inhibitory action of estrogen on testicular cord development (antagonistic effect) [56]. Fadrozole and letrozole had no apparent effect on testis differentiation [57].

In embryos incubated at 30 °C (female-producing temperature), estrogenic treatment somewhat reduced the size of the gonads but did not affect their differentiation into ovaries. Tamoxifen alone led to the development of ovotestes. A slight masculinization of the ovaries was still observed when estradiol benzoate was injected simultaneously with tamoxifen: epithelial cords were present in the medulla, although they were thinner than typical testicular cords [56]. Fadrozole and letrozole induced various degrees of gonadal masculinization, letrozole being more potent than fadrozole. In many

cases, ovotestes or testes with typical testicular cords were obtained [57, 58]. In such masculinized gonads, aromatase activity was significantly lower than in ovaries, being close or similar to that in testes of embryos incubated at 25 °C [58]. Treatment with Letrozole after TSP also resulted in different degrees of gonadal masculinization up to the formation of ovotestes, always exhibiting lower aromatase activity than that of an ovary. Thus, the ovary retains male potential after TSP [59].

Several data, similar to or completing those in *E. orbicularis*, were obtained in other reptilian species with TSD. Estrogenic treatment induced ovary differentiation at a male-producing temperature in all species studied, including crocodilian, lizard and other turtle species [60]. In *C. porosus*, specific binding of tritiated estradiol was found in the gonads of both sexes during the first stages of their sexual differentiation [61]. Accordingly, in the turtle *Trachemys scripta*, the time period during which gonadal differentiation is sensitive to exogenous estrogens was shown to coincide with the thermosensitive period [62]. Moreover, shifts from a male- to a female-producing temperature as well as estrogenic treatments at different stages of this period had similar chronological effects on gonadal structure, the inhibition of testicular cord development preceding the proliferation of germ cells in the cortex [39]. To obtain complete feminization of the gonads, the doses of exogenous estrogen required near the pivotal temperatures were lower than those required below this temperature, showing a synergistic effect of temperature and estrogens on gonadal differentiation [63].

Masculinization of gonads by an antiestrogen at a female-producing temperature was observed only in the turtle *E. orbicularis*. Thus, tamoxifen had an agonistic but not an antagonistic effect in embryos of *A. mississippiensis* [64] and *T. scripta* [65]. However, as in *E. orbicularis*, significant results were obtained with the aromatase inhibitors fadrozole and letrozole in two turtles, *T. scripta* and *Chelydra serpentina*. Fadrozole increased the percentage of males at both pivotal and female-producing temperatures in *T. scripta*, and at a predominantly female-producing temperature in *C. serpentina*. Likewise, in *T. scripta*, letrozole increased the percentage of males at a female-producing temperature [66–68].

Sex-reversed individuals (i.e. females resulting from treatment with an estrogen at a male-producing temperature and males resulting from treatment with an aromatase inhibitor at a female-producing temperature) are viable after hatching and display gonadal endocrine function similar to that of control males and females obtained under the effects of temperature alone. Thus estrogen-induced females of the leopard gecko (*Eublepharis macularius*) had normal circulating sex steroids and produced viable offspring as adults [69]. In 8-month-old turtles (*C. serpentina*), the profiles of circulating steroids (testosterone and 17 β-estradiol) and the response to treatment with follicle-stimulating hormone were similar in sex-reversed and temperature-induced males and females [70].

Altogether, the results of these experiments show that treatment with estrogens before or during TSP induces ovary differentiation at male-producing temperatures, whereas treatment with antiestrogens or aromatase inhibitors results in ovotestis or testis diffentiation at female-producing temperatures. It is therefore clear that estrogens are involved in ovary differentiation. Moreover, it appears that estrogens also play a role in maintaining the ovarian structure. In ovarian differentiation, estrogens act on both the inner part and the surface epithelium of the gonads. In the inner part, they inhibit differentiation of testicular cords from the initial epithelial cords (sex cords), and instead induce differentiation of lacunae bordered by a flat epithelium. At the surface, they stimulate the formation of a cortex from the initial germinal epithelium; cortex development is characterized by the proliferation of germ cells and their entry into meiosis. If estrogens are synthesized at very low levels, testicular cords differentiate, the cortex does not develop and testis differentiation occurs. The differential growth of gonads at a male- and a female-producing temperature follows these estrogenic effects [44].

Possible implication of androgens in gonadal differentiation

As indicated above, exogenous testosterone had no masculinizing effect at a female-producing temperature, but feminized the gonads at a male-producing temperature in *E. orbicularis* [34]. The same 'paradoxical' effect was obtained in *T. scripta* [67]. In both cases, it was expected to result from aromatization of testosterone. Aromatase activity is very low in testis. However, aromatization of exogenous testosterone could occur in other tissues, such as brain, which already exhibits aromatase transcription before TSP in both sexes [23]. Therefore, the effects of dihydrotestosterone (DHT), a nonaromatizable derivative of testosterone, were studied. Testosterone is converted to 5α-DHT or 5β-DHT through the activity of the 5α-reductase or the 5β-reductase. In *T. scripta* and *A. mississippiensis*, treatment with 5α-DHT had no detectable effect on gonadal differentiation at temperatures yielding 100% males or 100% females [65, 71]. Moreover, in *T. scripta*, 5α-reductase inhibitors did not prevent testis differentiation at a 100% male-producing temperature [67]. These results exclude a major role of androgens compared with that of estrogens [8]. However, when eggs of *T. scripta* were incubated at pivotal temperature, treatment with high dosages of

5α-DHT increased the percentage of males, whereas treatment with 5α-reductase inhibitors increased the percentage of females. Simultaneous administration of 5α-DHT and estradiol led gonads to develop as ovotestes, showing that 5α-DHT prevented the inhibitory effect of estradiol on testicular cord development [67]. A masculinizing effect of 5α-DHT in gonadal differentiation is therefore possible around the pivotal temperature, a condition which often occurs in reptile nests in nature [11].

In *E. orbicularis* embryos, both testes and ovaries produced in vitro 5α-pregnanes as the major metabolites of progesterone and 5α- androstanes as the major metabolites of dehydroepiandrosterone and androstenedione (fig. 3), showing a high and similar 5α-reductase activity in both sexes. However, testes but not ovaries produced 5α-DHT from dehydroepiandrosterone or androstenedione as precursors [49]. This can be simply a consequence of higher aromatase activity in ovaries (see fig. 3). In *D. coriacea*, both 5α- and 5β-reductase activities in gonads were high and similar at both male- and female-producing temperatures (G. Desvages and C. Pieau, unpublished results). Therefore, contrary to aromatase, the synthesis of 5α- and 5β-reductase in the gonads does not appear to be temperature-dependent in turtles with TSD.

The meaning of gonadal intersexuality

Long before the discovery of TSD, fertile testes with immature oocytes at their surface had been described in turtles captured in nature [72].

Recent data on *E. orbicularis* show that gonadal intersexuality occurs in different conditions of incubation and treatment: at, or close to, the pivotal temperature [28, 47]; at fluctuating temperatures in the laboratory [73] and in nature [11, 74]; under the effects of estrogenic treatment at a male-producing temperature [34, 56]; and under the effects of an antiestrogen or an aromatase inhibitor at a female-producing temperature [56–59].

In hatchlings of *E. orbicularis* incubated at the pivotal temperature (28.5 °C), ovaries have a typical structure, but most testes display an ovarian-like cortex with germ cells in meiosis. In some cases, the cortex is almost as developed as that of an ovary, and gonads are ovotestes [28, 47]. At 28.5 °C, the future testes and ovotestes become clearly distinct from the future ovaries only by the end of TSP, whereas the differences between a testis differentiating at 25 °C and an ovary differentiating at 30 °C are already detectable at the beginning of TSP [44].

During TSP, aromatase activity in the future testes and ovotestes at 28.5 °C remains low but slightly higher than in testes at 25 °C; in the future ovaries at 28.5 °C, aromatase activity increases but is generally slightly lower than in ovaries at 30 °C (fig. 4). At the end of embryonic development, aromatase activity in testes and ovotestes at 28.5 °C becomes similar to that in 25 °C testes, and aromatase activity in 28.5 °C ovaries becomes similar to that in 30 °C ovaries. During the same period, germ cells begin to degenerate in the cortex of ovaries and ovotestes. Germ cell degeneration continues after hatching. However, not all germ cells degenerate into ovaries, whereas in most cases all cortical germ cells degenerate into ovotestes, which thus become testes. Therefore, in general, the formation of an ovotestis is a transient phenomenon due to a slight increase in estrogen synthesis compared with that in a differentiating testis during TSP; estrogen levels are sufficient to induce the formation of an ovarian-like cortex but not sufficient to inhibit the development of the testicular cords. In addition, at the end of the embryonic life and after hatching, estrogen levels are

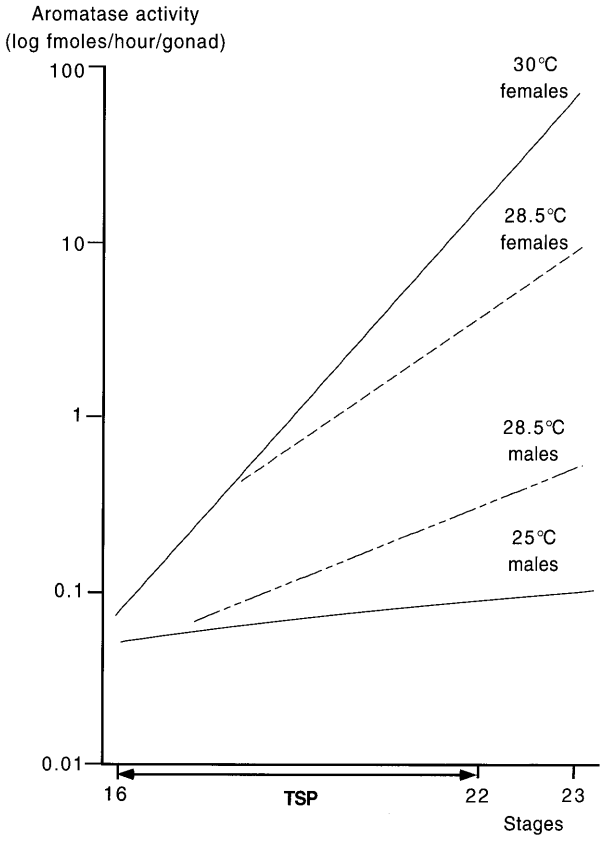

Figure 4. Gonadal aromatase activity during the TSP in *E. orbicularis* embryos incubated at 25 °C (male-producing temperature), 28.5 °C (pivotal temperature) and 30 °C (female-producing temperature). Gonadal aromatase activity in 28.5 °C males is slightly higher than in 25 °C males; in 28.5 °C females it is slightly lower than in 30 °C females (modified from [44]).

too low to maintain an ovarian-like cortex at the surface of the gonads. However, in a few individuals, some oocytes may escape degeneration, and several months after hatching, they are found in cortex vestiges at the surface of the testes [44].

Gonadal differentiation at the pivotal temperature in *E. orbicularis*, with transient formation of an ovarian-like cortex at the surface of the male gonad, appears very similar to that described 65 years ago in the turtle *Sternotherus odoratus* [30]. The eggs of this turtle were incubated at the ambient laboratory temperature, which probably was close to the pivotal temperature during TSP. Moreover, in this species as in *E. orbicularis*, oocytes at the surface of the testes were found in some individuals after hatching. Observations made in adult turtles show that testes can be functional (i.e. produce spermatozoa), although immature oocytes persist at their surface [72].

As shown above, experiments carried out in *E. orbicularis* with tamoxifen, fadrozole and letrozole at a female-producing temperature led to various degrees of gonadal masculinization [56–59]. The different types of intersexual gonads obtained ranging from a typical ovary to a typical testis had aromatase activity in accordance with their structure [58, 59]. Moreover, they confirmed that the epithelium of testicular cords and that of ovarian lacunae derive from the same lineage (i.e. proliferation of the germinal epithelium, see above). Different steps of transformation of ovarian lacunae into testicular cords or tubes were observed. The epithelium bordering lacunae is flat, whereas that of testicular cords is high (future Sertoli cells). In many cases, lacunae with both a flat epithelium and a high epithelium were found, reflecting subtle regulations between masculinizing and feminizing substances and somewhat different dose effects of these substances in each cell.

Genes involved or that could be involved in gonadal formation and differentiation

Molecular approaches of the mechanism of TSD in reptiles have recently been undertaken in two main directions: (i) cloning and expression of aromatase and estrogen receptor genes which both appear to play a central role in gonadal differentiation, and (ii) the identification and expression of homologues of genes that have been shown, or expected, to be involved in gonadal formation and/or differentiation in mammals.

Aromatase and estrogen receptor genes

Partial complementary DNA (cDNA) clones or full-length cDNAs of aromatase were isolated from two turtles, *Malaclemys terrapin* [75, 76] and *T. scripta* [21] and the alligator *A. mississippiensis* (V. A. Lance, unpublished results). In *M. terrapin*, aromatase transcript levels were measured using a competitive reverse transcription-polymerase chain reaction (RT-PCR) technique, in adrenal/mesonephros/gonad complexes, from the formation of genital ridges to hatching. At a male-producing temperature, aromatase transcript levels were below the detection limits of the assay at all stages, whereas at a female-producing temperature they increased exponentially during TSP [23, 77]. As expected, the profiles of aromatase expression in the adrenal/mesonephros/gonad complexes of *M. terrapin* parallels the profiles of aromatase activity in gonads of *E. orbicularis* [50]. Therefore, differential aromatase activity as a function of incubation temperature of embryos results from differential regulation of the aromatase gene at the transcription level.

Partial cloning of the cDNA and gonadal expression of the estrogen receptor were performed in the turtle *T. scripta* [21, 78]. Estrogen receptor messenger RNAs (mRNAs) were expressed in gonads of putative males and females as early as the beginning of TSP. In differentiating testes, transcripts were found in medullary testicular cords but not in the thin epithelium of the surface. In differentiating ovaries, transcripts were distributed throughout both the cortex and medulla [78]. These observations agree with the results of treatments with estrogens and antiestrogens, showing that estrogens act on both parts, the medulla and the cortex, of the gonad.

Homologues of mammalian genes

Two genes, *WT1* (Wilms's tumor 1) and *SF1* (steroidogenic factor 1, also known as Ad 4 BP, homologue of *fushi tarazu* factor 1, *FTZ-F1* from the fruit fly) are required for the formation of genital ridges in mammals [79].

Homologues of these two genes have been found in *T. scripta*. A cDNA clone for *WT1* of approximatively 2.7 kb was sequenced and shown to be 73.6% identical to human *WT1* at the nucleotide level. RNA was isolated from mesonephros/gonadal complexes and the brain of embryos incubated at a male- and a female-producing temperature, between the formation of genital ridges and the end of TSP. Northern blot analyses of RNA showed that at both temperatures, and at all stages, a single *WT1* transcript of approximately 3.2 kb was expressed in mesonephros/gonadal complexes but not in the brain [80]. Moreover, a putative cDNA clone for *SF1*, including the mammalian highly conserved regions I, II and III and the *FTZ-F1* box, was recently isolated from turtle adrenal/mesonephros/gonadal complexes [81].

Testis differentiation in mammals is governed by *SRY*. Two other genes are associated with differentiation of Sertolian cells: the *SRY*-related gene *SOX 9* and the *AMH* gene (anti-Müllerian hormone) [79].

Attempts to clone an *SRY*-like gene from reptiles revealed a whole family of *SOX* genes, 10 in the turtle *C. serpentina* [82], and 17 in *A. mississippiensis* [3]. None of these genes appeared to be sex-specific. However, recently, two cDNA clones for *SOX 9*, 3.9 kb and 2.2 kb, were obtained in *T. scripta*. The 2.2 kb clone was identical to the 3.9 kb clone except that it was truncated at both the 5′ and 3′ ends. Expression of *SOX 9* in mesonephros/gonadal complexes and in the brain of embryos incubated at a male- or a female-producing temperature was analyzed by Northern blots at different stages between the formation of genital ridges and the end of TSP. Two transcripts were found in these tissues at all stages, but during TSP the relative proportion of the two transcripts differed at the two temperatures. Whether the difference in the ratio of these transcripts reflects a true effect of temperature was not determined. Analysis of *SOX 9* expression was also carried out by in situ hybridization. Before and at the beginning of TSP, *SOX 9* was expressed in gonads at the two incubation temperatures; by the end of TSP, it was expressed in testes but no longer in ovaries. During all this period, *SOX 9* was expressed in mesonephros of both sexes [80].

A putative cDNA clone of *AMH* was also isolated recently in *T. scripta*. This cDNA is similar in size and GC content to chicken and mammalian *AMH* cDNAs, and the regions with the highest homologies between human and chicken are the same in *T. scripta* [81].

Data in mammals have shown the implication of *DAX1* (DSS-AHC critical region on the X, gene 1) in ovarian differentiation [79]. Recently, a partial sequence of *DAX1* homologue was isolated from an ovarian cDNA library in *A. mississipiensis* (V. A. Lance, unpublished results). It therefore appears that, except for *SRY*, all the genes that have been shown, or are expected, to be involved in mammalian gonadal differentiation have their homologues in TSD reptiles. Hence, several questions can be raised: Do homologues of mammalian sex-determining genes interact directly or indirectly with aromatase, and how? Is transcription of these genes, or of some of them, under estrogen control? How does temperature intervene in these processes?

A scheme presenting the possible mechanisms of the action of temperature resulting in the activation or the repression of transcription of the aromatase gene was presented previously [8]. Figure 5 includes in this scheme the newly identified factors/genes in TSD reptiles and presents some possible interactions with the aromatase gene and the estrogen-estrogen receptor complex. Transcription of the aromatase gene is under the control of activating and repressing factors.

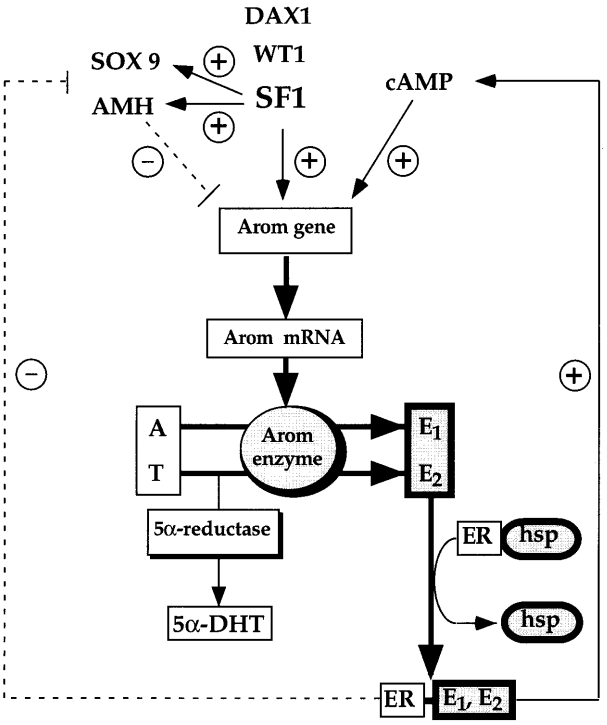

Figure 5. Possible interactions of homologues of mammalian sex-determining factors with the aromatase gene in TSD reptiles. ⊕, activation of transcription; ⊖ repression of transcription; A, androstenedione; E1, estrone; E2, estradiol-17β; ER, estrogen receptor; T, testosterone; DHT, dihydrotestosterone; hsp, heat-shock protein.

As shown in mammals, SF1 would act as a transcriptional activator of steroidogenic enzymes, including aromatase [83–85]. Moreover, SF1 would also be involved in the activation of expression of the genes *SOX 9* and *AMH*, both specific for testis differentiation, with amplification of this transcription when WT1 interacts with SF-1. As, on the other hand, AMH has been shown to repress aromatase gene transcription [86], we may consider that in TSD reptiles depending on the incubation temperature, SF1 would act either as a masculinizing factor (via SOX 9 and AMH) or as a feminizing factor (with direct or indirect activation of aromatase gene transcription). SOX 9 is expected to be an activator of *AMH* gene transcription in mammals and chicken [87, 88], although it does not seem to be involved in triggering this transcription in chicken [89].

In mammals, DAX1, by interacting with SF1, can prevent the synergistic action of WT1 and SF1 and act as a repressor of *SOX 9* and *AMH* transcription. Thus, by repressing the male pathway, DAX1 can be considered as a feminizing factor [90]. Has DAX1 the same func-

tion in TSD reptiles? How is it implicated in the activation of aromatase transcription by SF1?

Our model implies that transcription of masculinizing and feminizing genes or of some of them is under the control of oestrogens, masculinizing genes being downregulated, whereas feminizing genes are upregulated by estrogens. In agreement with repression of masculinizing genes by estrogens, a sequence nearly identical to the consensus sequence of estrogen response elements (EREs) has been found in the promoter region of the *AMH* gene in human and chicken [89, 91]. Repression of *SOX 9* gene transcription by estrogens deserve consideration, since in differentiating ovaries of *T. scripta*, *SOX 9* transcripts disappear [80] when aromatase expression strongly increases [77]. On the other hand, a positive feedback effect of estrogens would easily explain the amplification of aromatase synthesis/activity in differentiating ovaries [8].

At present, there is no evidence of a putative target for temperature. Therefore, the three possible mechanisms of action of temperature described in the previous model [8] can be considered in the new model presented here. Temperature could activate or repress synthesis of either a feminizing factor, a masculinizing factor, or the heat shock proteins involved in the binding of estrogens to estrogen receptors; temperature could also be implicated in the dissociation of hsp(s) from the complex estrogen-estrogen receptor which is then activated [8].

Salient points

To date, TSD has been demonstrated in crocodilians, turtles and lizards but not in snakes. During early stages of gonadal development, cells proliferate from the germinal epithelium, and aggregate into epithelial cords in the underlying mesonephric mesenchyme. Then, during the thermosensitive period and depending on the level of endogenous estrogens, these medullary cords yield in testes anlagen of seminiferous cords with high Sertolian epithelium, and in ovaries lacunae with flat epithelium. The estrogen level remains low in testes, whereas it strongly increases and stimulates development of the cortex in ovaries.

Aromatase is therefore a key enzyme in gonadal sex differentiation. Regulation of aromatase gene transcription is multifactorial and could be, directly or indirectly, influenced by temperature during the thermosensitive period.

The cDNAs corresponding to *WT1*, *SFI*, *SOX 9*, *AMH* and *DAX1* homologues of mammalian sex-determining factors have been characterized in TSD reptiles. In mammals, SF1 has been demonstrated to activate and AMH to repress aromatase gene transcription. Since SF1 also activates AMH gene transcription, could this factor not have a pivotal role in aromatase gene regulation? The search for other factors that are involved in gonadal steroidogenesis in mammals, such as the steroidogenic acute regulatory (StAR) protein [92] would be of particular interest also in TSD reptiles.

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