REVIEW

Oestrogens and temperature-dependent sex determination in reptiles: all is in the gonads

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Abstract

In many species of oviparous reptiles, the first steps of gonadal sex differentiation depend on the incubation temperature of the eggs. Feminization of gonads by exogenous oestrogens at a male-producing temperature and masculinization of gonads by antioestrogens and aromatase inhibitors at a female-producing temperature have irrefutably demonstrated the involvement of oestrogens in ovarian differentiation. Nevertheless, several studies performed on the entire gonad/adrenal/mesonephros complex failed to find differences between male- and female-producing temperatures in oestrogen content, aromatase activity and *aromatase* gene expression during the thermosensitive period for sex determination. Thus, the key role of aromatase and oestrogens in the first steps of ovarian differentiation has been questioned, and extragonadal organs or tissues, such as adrenal, mesonephros, brain or yolk, were considered as possible targets of temperature and sources of the oestrogens acting on gonadal sex differentiation.

In disagreement with this view, experiments and assays carried out on the gonads alone, i.e. separated from the adrenal/mesonephros, provide evidence that the gonads themselves respond to temperature shifts by modifying their sexual differentiation and are the site of aromatase activity and oestrogen synthesis during the thermosensitive period. Oestrogens act locally on both the cortical and the medullary part of the gonad to direct ovarian differentiation. We have concluded that there is no objective reason to search for the implication of other organs in the phenomenon of temperature-dependent sex determination in reptiles. From the comparison with data obtained in other vertebrates, we propose two main directions for future research: to examine how transcription of the *aromatase* gene is regulated and to identify molecular and cellular targets of oestrogens in gonads during sex differentiation, in species with strict genotypic sex determination and species with temperature-dependent sex determination.

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Introduction

Many species of oviparous reptiles, including crocodilians, a majority of turtles, some lizards and the two closely related species of Sphenodon have been shown to display temperature-dependent sex determination (TSD). In these species, the differentiation of gonads into ovaries or testes depends on the incubation temperature of the eggs during a critical period of embryonic development designated the thermosensitive period (TSP). Various treatments prior to and/or during this period have demonstrated the involvement of oestrogens in gonadal sex differentiation. Treatments with exogenous oestrogens result in ovarian differentiation at a male-producing temperature, whereas treatments with antioestrogens or aromatase inhibitors result in testicular differentiation at a female-producing temperature (for review see Pieau *et al.* 1999).

Several studies have sought for oestrogen content, aromatase activity and *aromatase* gene expression in the gonad/adrenal/mesonephros (GAM) complexes during the embryonic development of turtles and crocodilians (White & Thomas 1992*a*,*b*, Smith & Joss 1994*a*, Smith *et al.* 1995, Jeyasuria & Place 1997, 1998, Willingham *et al.* 2000, Gabriel *et al.* 2001, Murdoch & Wibbels 2003). In only one of these studies were differences between male- and female-producing temperatures found during the TSP (Jeyasuria & Place 1998). In the other studies, differences were found only at the end of the TSP and/or after the TSP, or were not found during and after the TSP. Therefore, some authors inferred that oestrogens are not involved in the early steps of ovarian differentiation (Smith & Joss 1994*a*, Smith *et al.* 1995, Murdoch & Wibbels 2003). Other authors inferred that temperature acts on extragonadal tissues, in particular the adrenal and/or

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mesonephros (White & Thomas 1992*a*), brain (Jeyasuria & Place 1998, Salame-Mendez *et al.* 1998, Gutierriez-Ospina *et al.* 1999, Willingham *et al.* 2000, Place *et al.* 2001) and yolk (Conley *et al.* 1997, Janzen *et al.* 1998, Bowden *et al.* 2000, 2002, Elf *et al.* 2002, for review see Elf 2003) since these tissues could be the sources of the oestrogens acting on gonadal sex differentiation.

We have analysed data obtained from the gonads alone, in particular in freshwater and marine turtles, that do not support the involvement of extragonadal tissues. This analysis has shown that (1) temperature has a direct effect on the gonads for their sexual differentiation, (2) during the TSP, aromatase activity in the gonads is dependent on the incubation temperature of the eggs, (3) oestrogens are synthesized by the gonads during the TSP and gonadal oestrogen synthesis depends on the incubation temperature of the eggs, and (4) oestrogens act on both the cortical and the medullary parts of the gonads.

Taking into account these data, we attempt to place the phenomenon of TSD in the larger context of gonadal sex differentiation in vertebrates and propose avenues for future research.

Key morphological events in turtle gonads during embryonic development

Most data on the gonads alone were obtained in the freshwater turtle, *Emys orbicularis* and two marine turtles, *Lepidochelys olivacea* and *Dermochelys coriacea*. Figures 1 and 2 illustrate the main morphological events occurring in the gonads during embryonic development in *E. orbicularis*. In this species, 25 °C yields 100% males, 30 °C yields 100% females and 28·5 °C (pivotal temperature) yields males, females and intersexes at hatching (Pieau 1976, Raynaud & Pieau 1985). In intersexes, the gonads are ovotestes (Fig. 2F). After hatching, ovotestes generally evolve as typical testes. However, some oocytes may persist at the surface of gonads through to adulthood (Pieau *et al.* 1998).

Based on the developmental stages defined in another freshwater turtle, *Chelydra serpentina* (Yntema 1968), the TSP extends between stages 16 and 22 of embryonic development in *E. orbicularis* (Pieau & Dorizzi 1981). Prior to the TSP, the gonads are indifferent (or bipotential) (Fig. 1). They are composed of the germinal epithelium and, in the inner part, of thin cords of epithelial cells (the so-called 'sex-cords') within loose mesenchymal tissue (Fig. 2A). At 25 °C, the first histological signs of differentiation of testicular cords (future seminiferous cords) appear at stage 17 (Figs 1 and 2B). During the TSP, the germinal epithelium flattens, and germ cells leave it to migrate between the epithelial cells (future Sertoli cells) of the testicular cords. At hatching, testicular cords are wide and display high Sertolian epithelium, whereas the surface epithelium is generally very thin (Fig. 2D). However, some remnants of the germinal epithelium enclosing germ cells may persist at the surface of testes. At 30 °C, in differentiating female gonads, during the TSP the initial sex-cords become thin, appear fragmented, or evolve as small lacunae bordered by a flat epithelium, whereas the germinal epithelium evolves as an ovarian cortex. Cortex development is mainly due to the *in situ* proliferation of germ cells. By the end of the TSP, some germ cells have entered into meiosis (Figs 1 and 2C). At hatching, primordial follicles with a growing oocyte are formed in the internal side of the cortex, in contact with the ovarian medulla (Figs 1 and 2E).

Gonadal development in other freshwater turtles is very similar to that in *E. orbicularis*. In marine turtles, it is roughly similar, although displaying some specificity. Thus, in *L. olivacea* and *D. coriacea*, germ cells have not entered into meiosis at the end of embryonic development (for review see Pieau *et al.* 1999). Developmental stages in embryos of marine turtles have been defined by Miller (1985). The correspondence of key stages with those defined in freshwater turtles is shown in Fig. 1.

Temperature has a direct effect on the gonads for their sexual differentiation

The direct effect of temperature on the gonads was demonstrated by experiments carried out *in vitro* in the marine turtle *L. olivacea* (Moreno-Mendoza *et al.* 2001). In this species, eggs incubated at 26 °C yield males, whereas eggs incubated at 33 °C yield females. Gonadal masculinization was characterized by the expression of the SOX9 protein during normal (*in ovo*) embryonic development. From stages 21 to 24 of embryonic development (see Fig. 1 for stages in marine turtles), SOX9 is expressed at both temperatures, in gonads which are still histologically undifferentiated. Thereafter, SOX9 remains positive at 26 °C in the medullary cords of differentiating male gonads, whereas it is downregulated at 33 °C in differentiating female gonads (Moreno-Mendoza *et al.* 1999).

The effects of temperature shifts on the expression of SOX9 were compared in gonads cultured *in vitro* and in gonads of whole embryos maintained *in ovo*. The paired gonads of embryos were explanted at stage 24 of development at 33 °C (Fig. 3 series 1A) and at stage 23 of development at 26 °C (Fig. 3 series 1B). One gonad of each pair was cultured at 33 °C and the contralateral one at 26 °C. Immunological detection of SOX9 was performed after different times of culture. SOX9 expression was maintained in gonads cultured at 26 °C, whereas it was downregulated in gonads cultured at 33 °C, following the same time-course as that in gonads of whole embryos submitted to the same thermal treatments. Thirteen days after explantation (Fig. 3 series 1B) the gonads cultured at 26 °C presented well-developed medullary cords expressing SOX9, whereas the gonads cultured at 33 °C presented an ovarian cortex and fragmented medullary cords without SOX9-positive cells.

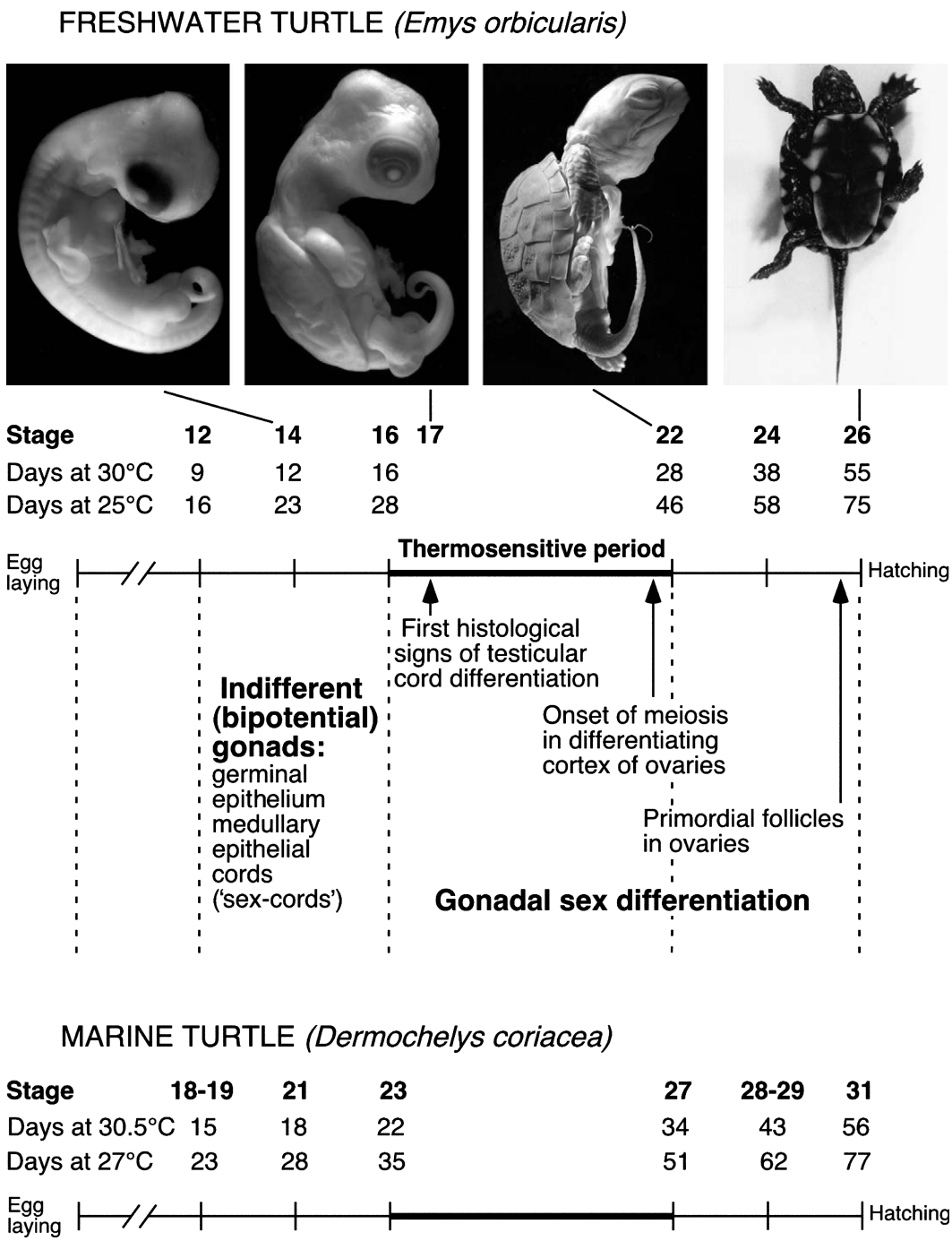

Figure 1 The main steps in the differentiation of gonads in relation to the age and developmental stage in embryos of the freshwater turtle *E. orbicularis*. Corresponding ages and stages in the marine turtle *D. coriacea* (according to Renous *et al.* (1989) and Desvages *et al.* (1993)).

In another series of experiments, gonads from embryos incubated at 33 °C were explanted at stage 24, cultured at 33 °C and shifted to 26 °C at different times; they were then cultured at 26 °C for 9 or 10 days (Fig. 3 series 2). When the shift was performed at 0, 2 and 3 days, SOX9 expression was maintained, but at day 4 SOX9 expression

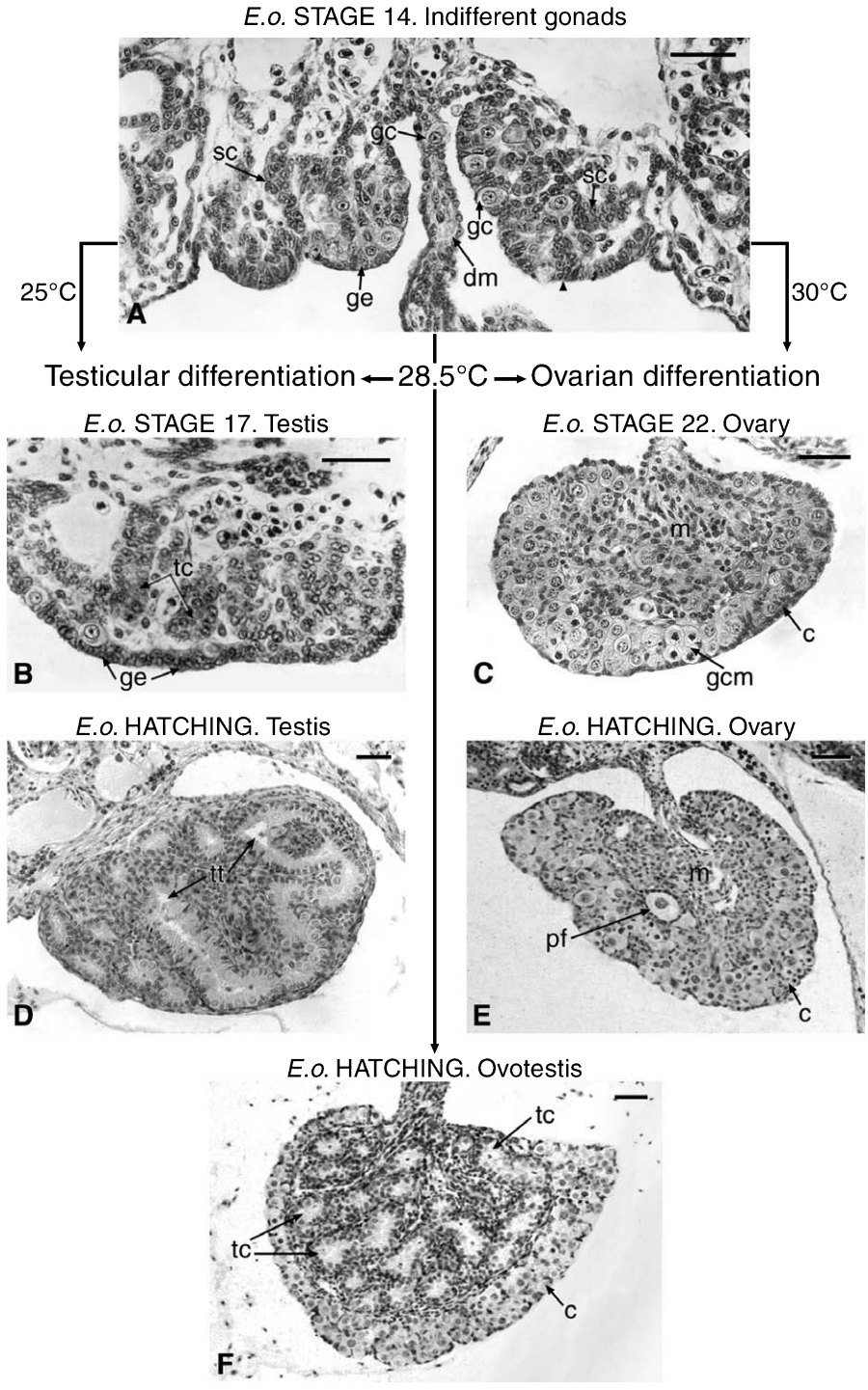

Figure 2 Structure of gonads at different stages of embryonic development at 25 °C (male-producing temperature), 30 °C (female-producing temperature) and 28·5 °C (pivotal temperature) in *E. orbicularis* (E.o). The arrowhead in (A) points to cell proliferation of the germinal epithelium that gives rise to the epithelial cords ('sex-cords') in the inner part of the gonad (c, cortex; dm, dorsal mesentery; gc, germ cell; gcm, germ cell in meiosis; ge, germinal epithelium; m, medulla; pf, primordial follicle; sc, sex-cord; tc, testicular cord; tt; testicular tube). Bars = 50 μm. (A–C) were previously published by Pieau (1974) and (E and F) in Raynaud & Pieau (1985).

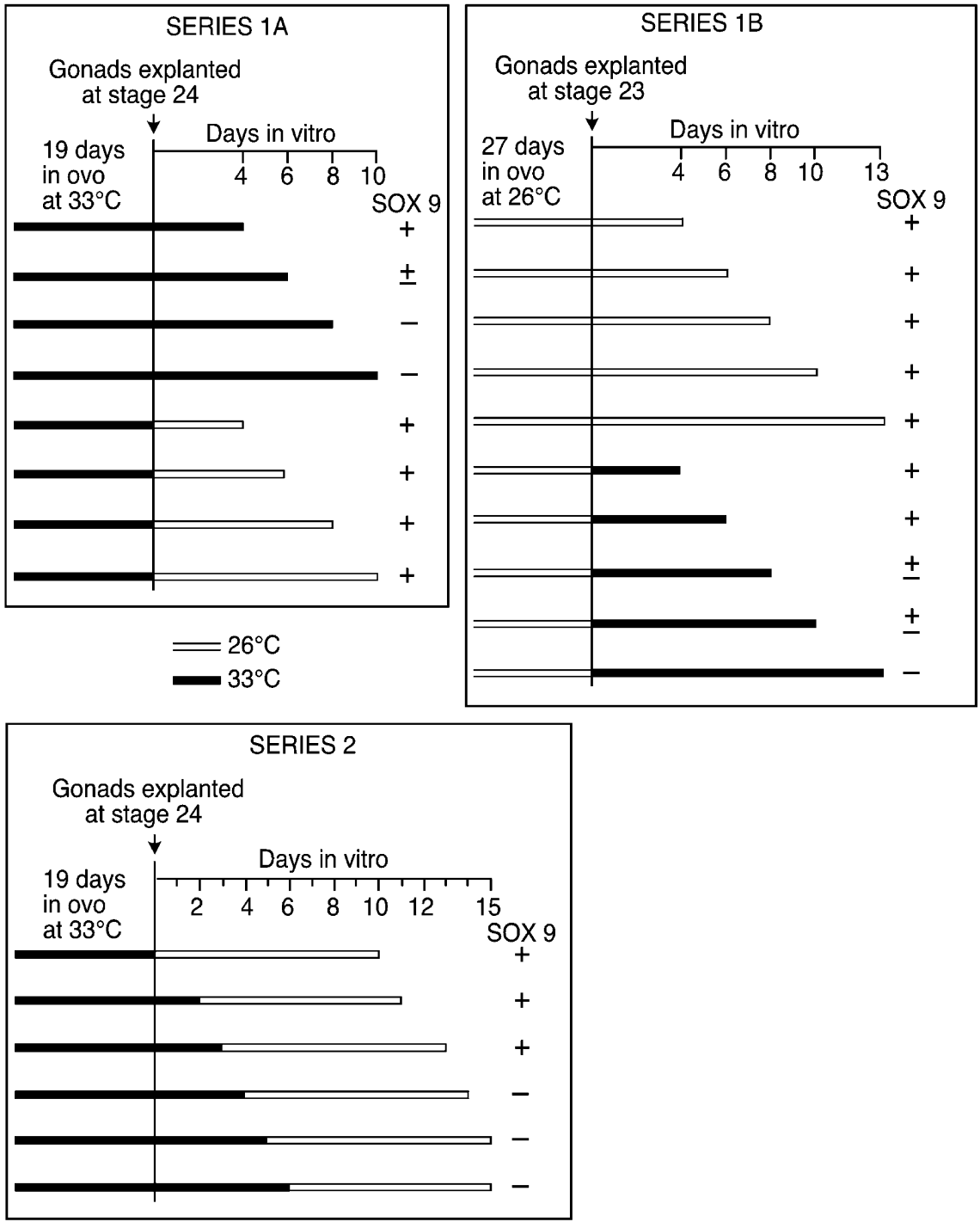

Figure 3 Effects of temperature shifts on SOX9 protein expression in gonads cultured *in vitro* at stages 24 and 23 of embryonic development in the marine turtle *L. olivacea*. Series 1A, 1B and 2 correspond to three distinct experiments (adapted from Moreno-Mendoza *et al.* (2001)).

was no longer observed in cultured gonads, indicating the commitment of ovarian differentiation after 4 days of culture at the female-producing temperature. Again the response was similar to that of control gonads of whole embryos shifted from 33 to 26 °C at the same ages (Moreno-Mendoza *et al.* 2001).

The similarity of response to temperature shifts in gonads cultured *in vitro* and in gonads developing in whole embryos clearly showed a direct effect of temperature on the gonads for their sexual differentiation.

During the TSP aromatase activity in the gonads depends on the incubation temperature of the eggs

Aromatase is the enzyme complex that converts androgens to oestrogens. Aromatase activity was measured in the gonads separated from the adrenal/mesonephros complexes in the freshwater turtle *E. orbicularis* and the marine turtle *D. coriacea*.

In *E. orbicularis* the TSP extends between stages 16 and 22 (Fig. 1). In a first series of experiments, aromatase activity was measured in pools of gonads from embryos incubated at 25 °C (male-producing temperature) or at 30 °C (female-producing temperature). At the beginning of the TSP, the aromatase activity was very low at both temperatures but was somewhat higher at 30 °C than at 25 °C. It thereafter remained low in differentiating testes at 25 °C, whereas it increased exponentially during the TSP and formed a peak by the end of embryonic development in differentiating ovaries at 30 °C (Desvages & Pieau 1992*a*). The same profiles were obtained by measuring aromatase activity in the paired gonads of each individual. In addition, by this method it was shown that during the TSP aromatase activity in testes and ovotestes at 28·5 °C (pivotal temperature) was slightly higher than in testes at 25 °C, and aromatase activity in ovaries at 28·5 °C was slightly lower than in ovaries at 30 °C (Pieau *et al.* 1998).

In a second series of experiments, aromatase activity was measured in gonads of embryos of *E. orbicularis* shifted from a male- to a female-producing temperature and vice versa at different stages of embryonic development and for different times. Shifts performed during the TSP resulted in an increase or a decrease of aromatase activity, whereas shifts after the TSP were ineffective (Desvages & Pieau 1992*a*). In addition, these experiments showed that the temperature acts on the regulation of aromatase synthesis rather than on the protein activity itself (Desvages & Pieau 1992*b*).

In *D. coriacea* eggs incubated below 29 °C yield males, whereas eggs incubated above 29·5 °C yield females (Rimblot *et al.* 1985). As shown above, the equivalents of stages 16 and 22 (delimiting the TSP) in *E. orbicularis* are stages 23 and 27 in *D. coriacea* (Fig. 1). Eggs of *D. coriacea* were shifted from 27 °C (masculinizing) to 35 °C (highly feminizing) at different embryonic stages between stages 22 and 29, and then incubated for 6 days at 35 °C. Only shifts performed between stages 23 and 27 resulted in a significant increase of gonadal aromatase activity compared with that in embryos maintained at 27 °C (Desvages *et al.* 1993). It is noteworthy that in *L. olivacea*, changes in expression of SOX9 protein resulting from temperature shifts were obtained during the same period (Moreno-Mendoza *et al.* 2001, see above). Therefore, the sensitive period to temperature shifts that induce changes of gonadal aromatase activity in *D. coriacea* and of SOX9 expression in *L. olivacea* coincides with the TSP for gonadal sex differentiation in *E. orbicularis*.

Differences between aromatase activities at male- and at female-producing temperatures were not seen during the TSP in the entire GAM complexes of *Crocodylus porosus* (Smith & Joss 1994*a*), *Alligator mississippiensis* (Smith *et al.* 1995) and *Trachemys scripta* (Willingham *et al.* 2000). Aromatase activity in the gonads was probably masked by that in the adrenal/mesonephros. Indeed, during the TSP, the gonad represents a very small portion of the GAM complex and, as shown in *E. orbicularis*, some aromatase activity is also present in the adrenal/mesonephros (Richard-Mercier *et al.* 1995). For the same reason, using RT-PCR, differences in *aromatase* mRNA levels between male and female GAMs were not seen during the TSP in *A. mississippiensis* (Gabriel *et al.* 2001) and *T. scripta* (Murdoch & Wibbels 2003). Therefore, extrapolating to the gonads data obtained for the entire GAMs may lead to misinterpretations.

Oestrogens are synthesized by the gonads during the TSP and gonadal oestrogen synthesis depends on the incubation temperature of the eggs

Using a histochemical method, Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) activity was found to be present in gonads of *E. orbicularis* already at stage 15, showing that steroidogenesis begins very early, prior to the TSP, in the indifferent gonads (Fig. 1). At this stage and at later stages, 3β-HSD activity was higher at the male- than at the female-producing temperature (Pieau 1974).

A study of steroid metabolism performed during and after the TSP confirmed this preliminary result and, in addition, showed that in the gonads all active enzymes involved in complete steroidogenesis are present during the TSP (Desvages & Pieau 1991).

Sensitive methods were used to measure gonadal oestrogen content. Assays were performed in embryos of *E. orbicularis* (Dorizzi *et al.* 1991) and *D. coriacea* (Desvages *et al.* 1993). In both species, the gonads were separated from the adrenal/mesonephros complexes and pooled. In *E. orbicularis*, detection of oestrogens (oestrone and oestradiol-17β) was carried out with a sensitive radioimmunoassay at stages 16, 17 and 18, i.e. at the beginning of the TSP. At stage 16, very low amounts of oestrogens were found at both male-producing temperature (25 °C) and female-producing temperature (30 °C). At stages 17 and 18, oestrogens remained detectable in very low amounts or were not detectable at the male-producing temperature, whereas higher levels of oestrogens were found at the female-producing temperature (Dorizzi *et al.* 1991).

In *D. coriacea*, detection of oestrogens was performed using an enzymatic method (Nicolas *et al.* 1979), at stages 26 (during the TSP) and 31 (hatching). At both stages, oestrogens were detected at the female-producing temperature (30·5 °C) but not at the male-producing temperature (27 °C). As expected, their levels at 30·5 °C, higher at stage 31 than at stage 26, were well correlated with aromatase activity (Desvages *et al.* 1993).

Given that the gonads synthesize oestrogens as early as at the beginning of the TSP and that gonadal oestrogen synthesis depends on the temperature during this period, it can be assumed that gonadal endogenous oestrogens but not extragonadal oestrogens – synthesized in the brain or contained in the egg yolk – are involved in TSD.

Oestrogens act on both the cortical and the medullary parts of the gonads

In reptiles at the end of embryonic development, the surface epithelium of the testes is generally very thin, but remnants of the initial germinal epithelium enclosing germ cells may persist locally. Previous early studies reported that oestrogenic treatment of immature alligators (*A. mississippiensis*, Forbes 1938) and newly hatched or juvenile turtles (*Malaclemys terrapin*, Risley 1941, *Mauremys leprosa*, Vivien & Stéfan 1958) induced proliferation and entry into meiosis of germ cells in remnants of the germinal epithelium. These results indicated that the development of an ovarian cortex from the germinal epithelium is controlled by oestrogens. However, after such late treatments, testicular cords were maintained in the medullary part of the gonads.

At the time of the discovery of TSD in turtles, oestradiol was injected into eggs incubated at a male-producing temperature in two species, *Testudo graeca* and *E. orbicularis*. In *T. graeca*, treatments performed early – when embryonic gonads still appeared histologically undifferentiated – inhibited development of testicular cords (they became thin or lacunar), but not later treatments (Pieau 1970). In *E. orbicularis*, treatments were performed at stages 19–20 (i.e. during the TSP) with different doses of oestradiol. In general, the higher the doses of hormone, the stronger the inhibition of testicular cord development. In all cases, an ovarian cortex was formed. Thus, the gonads of treated embryos were typical ovaries, hypo-ovaries (with a very reduced medullary part) or ovotestes (Pieau 1974).

Since then, ovary differentiation at a male-producing temperature under the effects of oestrogenic treatment has been obtained in all other reptilian species studied, including crocodilian, lizard and turtle species (Bull *et al.* 1988, Pieau *et al.* 1994*a*). The time-period during which the treatment is efficient coincides with the TSP (Gutzke & Chymiy 1988). During this period, binding of tritiated oestradiol by the gonads was shown to occur at both male- and female-producing temperatures (Smith & Joss 1994*b*). Moreover, *oestrogen receptor* mRNAs were detected in putative male and female gonads as early as at the beginning of the TSP. Transcripts were distributed throughout the cortex and medulla in differentiating ovaries, whereas they were found in medullary testicular cords but not in the flattened parts of the surface epithelium in differentiating testes (Bergeron *et al.* 1998). However, as shown above, the development of an ovarian-like cortex under the effects of exogenous oestradiol remains possible after the TSP in the remnants of the germinal epithelium, indicating the persistence of oestrogen receptors in these regions.

In the freshwater turtle *T. scripta*, shifts from a male- to a female-producing temperature as well as oestrogenic treatments at the same embryonic stages during the TSP had similar chronological effects on gonadal structure, i.e. inhibition of testicular cord development preceding the proliferation of germ cells in the cortex (Wibbels *et al.* 1993). It can therefore be assumed that, at a female-producing temperature, oestrogens synthesized by the gonads themselves (see above) direct their differentiation into ovaries.

The implication of oestrogens in ovary differentiation has been irrefutably confirmed by the results of treatments with antioestrogens and aromatase inhibitors prior to and/or during the TSP. It suffices to recall here that treatments with the antioestrogen tamoxifen in *E. orbicularis* and the aromatase inhibitors fadrozole or letrozole in *E. orbicularis*, *T. scripta* and *C. serpentina* resulted in differentiation of testicular cords in gonads at a female-producing temperature: at hatching, the gonads were testes or ovotestes (for review see Pieau *et al.* 1999). In *E. orbicularis*, treatment with letrozole somewhat after the TSP also resulted in masculinization of gonads but was considerably less efficient than during the TSP; not only were the masculinized individuals less numerous, but all these individuals displayed ovotestes (Dorizzi *et al.* 1996, Belaïd *et al.* 2001).

A characteristic of masculinization of gonads under the effects of aromatase inhibitors is the presence of lacunar tubes, or lacunae, bordered by a flat epithelium and a higher Sertolian-like epithelium. These structures were observed in *E. orbicularis* after treatment during the TSP (Dorizzi *et al.* 1994, Richard-Mercier *et al.* 1995) and also after the TSP (Dorizzi *et al.* 1996, Belaïd *et al.* 2001). They were also observed in the sex-reversed gonads of genetic female chickens treated with an aromatase inhibitor prior to sexual differentiation (Vaillant *et al.* 2001*a*). In these gonads, the thickened part of the epithelium of the lacunae was shown to express the anti-Müllerian hormone (*AMH*), *SOX9* and *SF1* mRNAs as does the Sertolian epithelium of control testicular cords, whereas the flattened part did not. Lacunae bordered by a flat epithelium are normally formed in the ovarian medulla. Thus, during sex reversal, ovarian lacunae evolve as testicular cords, by transdifferentiation of their epithelium into a Sertolian epithelium (Vaillant *et al.* 2001*b*). In *E. orbicularis*,

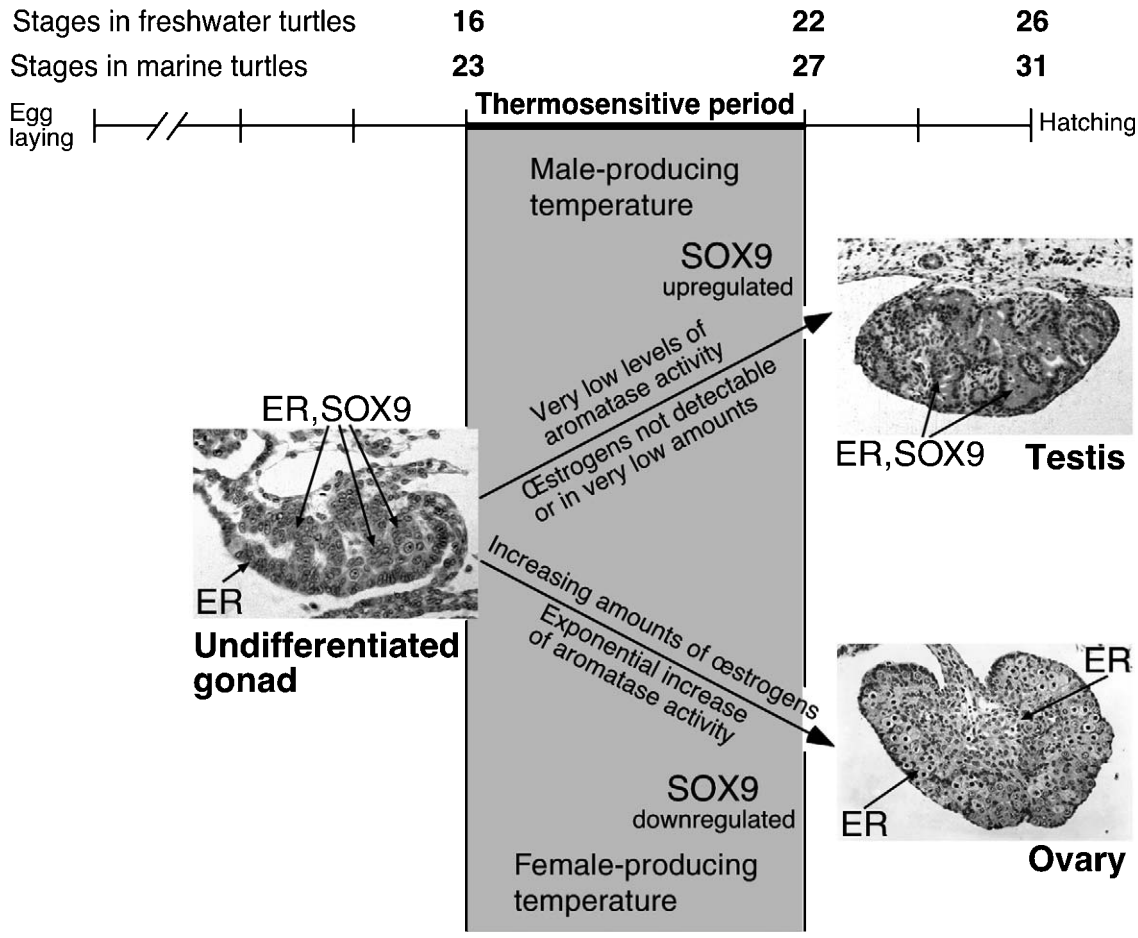

Figure 4 Molecular and physiological events in the gonads consistent with the involvement of endogenous oestrogens in the first steps of ovarian differentiation in freshwater and marine turtles (ER, oestrogen receptor).

testicular cords of ovotestes, resulting from treatment with letrozole, express *SOX9* mRNAs as testicular cords of control testes (Belaïd *et al.* 2004).

Conclusions and perspectives

Taken together, the data obtained from experiments and assays carried out on the gonads alone in turtle embryos provide evidence that, in reptiles exhibiting TSD, the gonads are the targets of temperature for their sexual differentiation and are the site of aromatase activity and oestrogen synthesis during the TSP. Gonadal oestrogen synthesis depends on the incubation temperature. At female-producing temperatures, as early as at the beginning of the TSP, endogenous oestrogens act locally on both the cortical and the medullary parts of the gonad to direct ovarian sex differentiation. The molecular and physiological events that support this major role of oestrogens are shown in Fig. 4.

In our opinion, contrary to the suggestion of Murdoch & Wibbels (2003), there cannot exist on one hand reptilian species such as *E. orbicularis*, *D. coriacea* and *M. Terrapin*, in which oestrogens are involved in ovarian sex differentiation as early as at the beginning of the TSP, and on the other hand reptilian species such as *C. porosus*, *A. mississippiensis* and *T. scripta*, in which oestrogens are involved in later stages of ovarian differentiation, i.e. only at the end or after the TSP. Using appropriate methods, aromatase activity, *aromatase* mRNA levels and oestrogen content in the gonads should be higher at the female- than at the male-producing temperature during the TSP in all reptilian species exhibiting TSD. Should this be otherwise, the results of treatments with exogenous estrogens and treatments with antioestrogens or aromatase inhibitors would be very difficult to interpret, as recognized by Murdoch & Wibbels (2003) themselves.

The thermosensitivity of gonadal sex differentiation has been demonstrated not only in many reptile species but also in several fish and some amphibian species. In

thermosensitive species, mitotic sex chromosomes are not distinguishable by classical caryological studies. However, a mechanism of genotypic sex determination (GSD) – male heterogamety, female heterogamety or polygenic – has been shown in these species by various methods, such as breeding tests after hormonal or thermal treatments, examination of lampbrush chromosomes or the electrophoretic pattern of sex-linked enzymes (fish: for review see Baroiller & Guiguen 2001, amphibians: for review see Chardard *et al.* 2004). Therefore, the effects of temperature are superimposed on the GSD mechanism.

As in reptiles, in fish and amphibians oestrogens are involved in ovarian differentiation, and aromatase plays a key role in this process. In the tilapia *Oreochromis niloticus*, the differentiating ovary displays a stronger expression of the *aromatase* gene and higher levels of oestradiol-17β than the differentiating testis (D'Cotta *et al.* 2001). In the newt *Pleurodeles waltl*, transcripts of the *aromatase* gene have been detected as early as at the beginning of the TSP in gonad/mesonephros complexes and their levels are higher in female gonads than in male gonads by the end of this period (Kuntz *et al.* 2003). It is therefore reasonable to assume that the same mechanism of action of temperature, resulting in the activation or the repression of transcription of the *aromatase* gene in gonads, exists in fish, amphibians and reptiles. Some possible mechanisms have been considered (Pieau 1996), but to date the molecular target of temperature remains unknown.

Oestrogens are also involved in ovarian differentiation of birds that all display a male ZZ/ female ZW mechanism of GSD (Elbrecht & Smith 1992). In chickens, *aromatase* transcripts have been detected in the two female gonads but not in the male gonads at day 6–6·5 of embryonic development, i.e. just prior to or at the very beginning of the first histological signs of gonadal sex differentiation (for review see Vaillant *et al.* 2001*a*). During the first steps of gonadal sex differentiation (days 7 and 8), *aromatase* gene expression is upregulated in female gonads while it remains undetectable in male gonads (Nishikimi *et al.* 2000, Oréal *et al.* 2002).

In marsupials, the implication of oestrogens in the first steps of ovarian differentiation can be expected since treatment of male pouch young with oestrogens leads to sex reversal of the testes: the gonads resemble ovotestes or ovaries with a clearly defined cortex containing germ cells having entered into meiosis (Burns 1961, Coveney *et al.* 2001). In eutherian mammals, *in vivo* treatment of gravid females with exogenous oestrogens does not lead to sex reversal of XY genetic male embryos (Greene *et al.* 1940). However, definitive evidence that oestrogens play a critical role in maintaining ovarian structure has been found recently. In XX genetic female mice with targeted disruption of the genes encoding oestrogen receptors α and β (ERαβKO mice) or aromatase (ArKO mice), Leydig cells differentiate from interstitial cells, and seminiferous tubule-like structures presenting Sertoli cells expressing *SOX9* differentiate from pre-existing granulosa cells after birth (Couse *et al.* 1999, Britt *et al.* 2002, Dupont *et al.* 2003). If ArKO female mice are fed a diet containing phyto-oestrogens, the appearance of Leydig and Sertoli cells is postponed and reduced. Likewise, if oestradiol-17β is administered to such females, the number of Leydig and Sertoli cells decreases in the ovaries (Britt *et al.* 2002, for review see Britt & Findlay 2003). From these findings, it may be asked whether, in eutherian mammals, oestrogens are not also involved in the first steps of ovarian differentiation.

A very simple scheme for the involvement of the *aromatase* gene and oestrogens in gonadal sex differentiation of vertebrates including mammals has been proposed previously (Pieau *et al.* 1994*b*). In accordance with this view and considering the evolutionary aspects, Crews (1994) estimates that 'vestigial remnants of a sex hormone mediation of sex determination might be present in mammals'.

Given the converging data obtained in non-mammalian vertebrates that provide evidence of a key role of aromatase and oestrogens in the first steps of ovarian differentiation and their possible implication in mammals, it seems to us that future research should be carried out in two main directions. The first direction should be to examine how transcription of the *aromatase* gene is regulated in gonads at the time of sex differentiation in species with strict GSD and species with TSD. The second direction should be to identify molecular and cellular targets of oestrogens in the cortical and the medullary parts of the gonads during sex differentiation. *A priori*, the same targets should be found in all vertebrates, whatever the mechanism of sex determination.

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