Sexual Differentiation of Gonads as a Function of Temperature in the Turtle *Emys orbicularis*: Endocrine Function, Intersexuality and Growth

CLAUDE PIEAU,* MIREILLE DORIZZI, NOELLE RICHARD-MERCIER, AND GISELE DESVAGES

Institut Jacques Monod, C.N.R.S. et Universités Paris 6 et 7, 75251 Paris Cedex 05, France

**ABSTRACT** *Emys orbicularis* is a freshwater turtle with temperature-dependent sex determination. Estrogens play a major role in gonadal differentiation; when they are produced at high levels during the thermosensitive period (TSP), ovaries differentiate; when their synthesis is very low, testes differentiate. Estrogens are synthesized from androgens through the activity of aromatase. We examine here two aspects of gonadal differentiation, intersexuality and growth, in *E. orbicularis*. For gonadal intersexuality, we studied the relationship between gonadal aromatase activity and gonadal structure at 28.5°C (pivotal temperature), from the beginning of TSP to hatching, and compared results to those obtained at 30°C (producing 100% females) and 25°C (producing 100% males). At 28.5°C, both males and females are obtained. However, histological differentiation of gonads is delayed compared to that at 25°C and 30°C, and an ovarian-like cortex of various thicknesses often develops at the surface of the male gonads; thus, several individuals display ovotestes at hatching. Despite important individual variations, the aromatase activity in ovaries differentiating at 28.5°C increases during development as in ovaries differentiating at 30°C. In most cases, however, activity is slightly lower than at 30°C, and at the end of embryonic life, it becomes similar to that at 30°C. In testes or ovotestes differentiating at 28.5°C, aromatase activity remains low but is generally slightly higher than in testes at 25°C; however, at the end of embryonic development, it becomes similar to that at 25°C. Oocytes in the cortex of ovotestes begin to degenerate around hatching and continue to degenerate after hatching. Therefore, ovotestes evolve as testes. However, some oocytes may persist at the surface of testes up to the adult age.

To estimate gonadal growth, the protein content was measured at different embryonic stages at 25°C and at 30°C. Testis growth is fast during TSP, somewhat slower after TSP, and decreases around hatching. Ovary growth is much slower than testis growth during TSP and then accelerates up to the end of embryonic development. This differential growth is well correlated with gonadal aromatase activity—much higher at 30°C than at 25°C—and can be explained by the fact that during TSP, testicular cords develop at 25°C whereas they are inhibited at 30°C; the ovarian cortex begins to form during this period but grows chiefly after TSP.

Both inhibition of testicular cord development and stimulation of cortex development are under the control of endogenous estrogens. In the case of ovotestes, slight increases in estrogen synthesis, compared to that in typical testes, are sufficient to induce the transient formation of an ovarian-like cortex although they do not inhibit the development of testicular cords. *J. Exp. Zool.* 281:400–408, 1998. © 1998 Wiley-Liss, Inc.

*Correspondence to: C. Pieau, Institut Jacques Monod, C.N.R.S. et Universités Paris 6 et 7, 2 place Jussieu, 75251 Paris Cedex 05, France. E-mail: pieau@ccr.jussieu.fr

Received 17 February 1998; Accepted 18 February 1998

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may be as developed as in an ovary; these gonads are ovotestes. In some individuals with ovotestes, the Müllerian ducts regress; in others, they are still complete at hatching although they are somewhat thinner than in females (Pieau, '76; Raynaud and Pieau, '85).

The effects of early treatments (before or/and during TSP) with estrogens at 25°C (a male-producing temperature), and with Tamoxifen, an antiestrogen, or nonsteroidal aromatase inhibitors such as Fadrozole or Letrozole at 30°C (a female-producing temperature) have shown the involvement of estrogens in ovary differentiation (Dorizzi et al., '91, '94). Estrogens have two effects: they inhibit testicular cord development and stimulate ovarian cortex formation (Dorizzi et al., '91). Later treatments (after TSP) with Letrozole have revealed that estrogens are also required to maintain the ovarian structure (Dorizzi et al., '96). All these different treatments yield several hatchlings with ovotestes similar to those observed within the transitional range of temperatures.

Estrogens are produced by conversion of androgens through the activity of the aromatase enzyme complex. The profiles of gonadal aromatase activity, established from measurements on pools of gonads at different embryonic stages, are very different at 25°C and at 30°C. In testes differentiating at 25°C, aromatase activity remains very low from the beginning of TSP to hatching. In ovaries differentiating at 30°C, aromatase activity is also very low at the beginning of TSP; however, it increases exponentially during TSP and peaks after TSP; there is a slight decrease of activity at hatching (Desvages and Pieau, '92a). Changes in gonadal structure resulting from temperature shifts during embryonic development or from treatments with an aromatase inhibitor are well correlated with changes in gonadal aromatase activity (Desvages and Pieau, '92b; Richard-Mercier et al., '95; Dorizzi et al., '96).

In the first part of this paper, we examine, from the beginning of TSP to hatching, the relationship between gonadal aromatase activity and gonadal structure at 25°C (temperature giving 100% males), 30°C (temperature giving 100% females), and 28.5°C (pivotal temperature). As a result of this study, we are able to propose a simple explanation, based on estrogen levels during TSP, for the origin of intersexuality in E. orbicularis. Moreover, in order to follow the evolution of intersexuality, we have studied the structure of gonads of individuals incubated at 28.5°C from the end of embryonic life to 9 months after hatching. We show that intersexuality is a transient phenomenon, because in most cases, ovotestes evolve as typical testes.

In the second part of the paper, we compare gonadal growth at 25°C and at 30°C from the beginning of TSP to hatching. Observed differential growth is well correlated with differential aromatase activity.

**GONADAL AROMATASE ACTIVITY RELATED TO GONADAL STRUCTURE**

This study was performed on 463 embryos (105 at 25°C, 152 at 30°C, and 206 at 28.5°C) from stage 16 (beginning of TSP) to stage 26 (hatching). To correlate aromatase activity and structure of the gonads, aromatase activity assay was carried out on the two gonads of each individual, using the same technique described previously (Richard-Mercier et al., '95). Gonads were then processed for histology.

Gonadal aromatase activity from the beginning of TSP to hatching is shown in Figure 1A and, with other ordinates, only during TSP in Figure 1B. The structure of the gonads at the end of TSP in embryos incubated at different temperatures is shown in Figure 2 and that of ovotestes and ovaries after TSP in individuals incubated at 28.5°C is shown in Figure 3.

**Incubations at 25°C and 30°C**

The first histological signs of testis differentiation at 25°C precede those of ovary differentiation at 30°C: they are distinguishable at stage 17 for the testis and at stage 19 for the ovary (Pieau, '74a). At stage 18, gonadal aromatase activity is already higher at 30°C than at 25°C (Fig. 1B). It then remains very low at 25°C and increases exponentially at 30°C (Fig. 1A), thus confirming previous observations (Desvages and Pieau, '92a). However, there are important individual variations. For example, at stage 25, aromatase activity at 30°C varies from 37 to 357 fmoles/hour/gonad! We have not found a clear correlation between aromatase activity and the volume of the cortex, the volume of the medulla, or the ratio volume of cortex/volume of medulla. Differences in aromatase activity thus appear to be mainly due to differences in the size of the gonads, the thinnest ones presenting the lowest aromatase activity.

**Incubation at 28.5°C**

At 28.5°C, gonadal differentiation is delayed compared to that at 25°C and 30°C. Indeed, in most individuals, there is no clear histological sign of such differentiation before the end of TSP. Thus,
at stage 21, gonads seem still undifferentiated: the medulla is relatively voluminous and contains thin epithelial cords, and, at the surface, the germinal epithelium persists or a cortex anlage is formed (Fig. 2A). At this stage, in gonads at 25°C, testicular cords are well-formed and the surface epithelium is flattened (Fig. 2B), whereas, in gonads at 30°C, the volume of the medulla is very reduced and a cortex anlage is formed (Fig. 2C).

At stage 22 (end of TSP), the differences between the future testes (or ovotestes) and the future ovaries developing at 28.5°C become obvious. In the male gonads, the medulla is voluminous and contains thin testicular cords and testicular tubes with a lacunal aspect; the germinal epithelium is still present (compare Fig. 2D and Fig. 2B). In the female gonads, the volume of the medulla is reduced and does not display testicular cords or tubes, and the cortex anlage is developing (Fig. 2E). At stage 23, most males exhibit testes with an ovarian-like cortex developing at the surface (Fig. 3A); germ cells in the cortex enter meiosis as they do in ovaries (compare Fig. 3A and Fig. 3B); in the medulla, testicular tubes are still la-
Fig. 2. Transverse sections through gonads of *Emys orbicularis* embryos by the end of TSP. After measurement of aromatase activity (aa, in fmoles/hour/gonad) gonads were processed for histology, i.e., fixed in Bouin’s fluid, embedded in paraffin, serially sectioned at 7.5 μm, and stained with hematoxylin and eosin. (A) Incubation at 28.5°C, stage 21: the inner part of the gonad is relatively voluminous and contains thin epithelial cords (ec), small lacunae bordered by a flat epithelium and groups of germ cells; the surface is covered by the germinal epithelium (ge); aromatase activity (aa = 0.26) indicates that this gonad would have evolved as an ovary. (B) Incubation at 25°C, stage 21: the gonad is a testis with already well differentiated testicular cords (tc) or tubes with a lacunal aspect (tt); the surface epithelium is thin (aa = 0.04). (C) Incubation at 30°C, stage 21: the gonad is much smaller than in A and B, because of an important reduction of the volume of the medulla, which is devoid of testicular cords; a cortex anlage (ca) indicates that this gonad is differentiating into an ovary (aa = 0.35). (D) Incubation at 28.5°C, stage 22: the gonad is differentiating into a testis, the medulla is voluminous and contains thin testicular cords (tc) or testicular tubes with a lacunal aspect (tt); at the surface, the germinal epithelium (ge) is maintained (aa = 0.23). (E) Incubation at 28.5°C, stage 22: the gonad is differentiating into an ovary, a cortex anlage (ca) is well formed and there are no testicular cords or tubes in the inner part (aa = 1.27). Bar, 50 μm.
cunal and many lacunae are bordered by a flat epithelium, not yet of the Sertolian type (Fig. 3A). At stage 25, testicular cords or tubes with a typical (high) Sertolian epithelium are well differentiated and in ovotestes the cortex is very similar to that in ovaries, except that it does not cover the entire surface of the gonads (compare Fig. 3C to Fig. 3D). At hatching, some oocytes are degenerating in the cortex of both ovotestes and ovaries; however, primordial follicles are formed in ovaries, whereas generally they are not formed in ovotestes (not shown).

Up to stage 19, it is not possible to identify the future males and females by the gonadal aromatase activity of embryos incubated at 28.5°C. However, as of stage 20, two categories of gonads can be distinguished with respect to aromatase activity (Fig. 1B), although it is difficult to distinguish gonadal sex by histology. In the first one (differentiating testes or ovotestes), the aromatase activity remains low during development, but in most cases it is slightly higher than in testes developing at 25°C (Fig. 1B); it is well correlated with cortex development up to stage 24, but not at later stages. Indeed, at the end of embryonic development (stages 25–26), the aromatase activity in ovotestes becomes similar to that in testes at 25°C. In the second category of gonads (differentiating ovaries), aromatase activity increases strongly during development, although it displays important individual variations as in incubations at 30°C. The aromatase activity in ovaries developing at 28.5°C is clearly higher than that in testes and ovotestes but is generally slightly lower than that in ovaries developing at 30°C. At the end of embryonic development, ovarian aromatase activity at 28.5°C becomes similar to that at 30°C (Fig. 1A).

**STRUCTURAL EVOLUTION OF GONADS OF INDIVIDUALS INCUBATED AT 28.5°C**

The evolution of gonads was followed for 9 months after hatching in individuals that had been previously typed for serological H-Y antigen expression in blood (Zaborski et al., '88). At the time of hatching, ovaries and ovotestes are infiltrated by aggregates of lymphocyte-like cells and apoptotic cells, which penetrate deeply into the gonads, up to the cortex, and seem to be associated with germ-cell degeneration. This phenomenon had already been observed in another turtle, *Chelydra serpentina* (Yntema, '81). It will be described in detail elsewhere for *Emys orbicularis*. Not all germ cells degenerate into ovaries, whereas almost all cortical germ cells degenerate after hatching into ovotestes; therefore, ovotestes become typical testes. However, some oocytes may escape degeneration, and nine months after hatching, they are found in cortex vestiges at the surface of the gonads of some individuals (Fig. 4E). Nevertheless, the biggest oocytes in ovotestes are much smaller than those in ovaries (compare Fig. 3E and Fig. 3F).

**GONADAL GROWTH AT 25°C AND AT 30°C**

Gonadal growth has been estimated by measuring the protein content at different stages of embryonic development, from the beginning of TSP to hatching (Fig. 4). Protein determinations were performed on pools of gonads of the same embryonic stage by the Coomassie microassay (Pierce).

There is a striking difference between testicular growth at 25°C and ovarian growth at 30°C. At 25°C, the protein content of the gonads increases continuously and strongly from stage 16 (0.7 μg/gonad) to stages 22–23 (8–11 μg/gonad); it increases less rapidly between stages 23 and 25 (10–12 μg/gonad) and decreases somewhat around hatching. At 30°C, at stage 16, the gonadal protein content is already slightly lower than at 25°C. It increases during TSP but much more slowly than at 25°C. Between stages 21 and 23, gonadal growth even appears inhibited, the slope at 30°C being four times lower than at 25°C. After stage 23, ovarian protein content increases again strongly to reach that in testes at stage 25 and is maintained at the same level (10–12 μg/gonad) up to hatching. Therefore, ovaries grow more slowly than testes during the first stages of gonadal differentiation, mainly during TSP. Figures 2B and 2C illustrate the difference in size of the

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**Fig. 3.** Transverse sections through gonads of *Emys orbicularis* individuals issued from eggs incubated at 28.5°C. Histological procedures are as detailed in the legend of Figure 2. For gonads shown in A–D, aromatase activity (aa in fmol/hour/gonad) was measured. (A) Ovotestis, stage 23: the medulla contains testicular tubes with a lacunal aspect (tt) and lacunae bordered by a flat epithelium; an ovarian-like cortex (olc) with germ cells in meiosis is formed (aa = 0.48). (B) Ovary, stage 23: a thick cortex (c) covers the ventral and lateral sides of the gonad (aa = 5.45). (C) Ovotestis, stage 25: typical testicular tubes (tt) with high Sertolian epithelium are now present in the medulla and most germ cells are in meiosis in the ovarian-like cortex (olc) (aa = 1.61). (D) Ovary, stage 25: the cortex (c) is thick and the medulla is reduced (aa = 125.83). (E) Ovotestis, young male at 9 months: the gonad contains well-delineated testicular tubes (tt); however, there are still vestiges of a cortex with some growing oocytes (oo). (F) Ovary, young female at 9 months: the cortex (c) contains oocytes (oo) much bigger than those persisting at the surface of ovotestes (compare with Fig. 3E). Bar, 50 μm.
two types of gonads at the end of TSP; at this stage, there is an average of 7 µg of protein per testis, whereas the mean content per ovary is 3 µg of protein only (Fig. 4).

Differential gonadal growth depending on the temperature is in agreement with the patterns of both gonadal development and aromatase activity. At 25°C, the continuous growth is due to the development of testicular cords or tubes, which starts at the beginning of TSP. As aromatase activity (Fig. 1) and therefore estrogen levels remain very low, testicular cord differentiation occurs. The high protein content between stages 23 and 25 corresponds to a period during which the Sertolian epithelium of testicular cords/tubes is particularly high and appears very active; Müllerian ducts regress at these stages (Pieau, '74a). At 30°C, the medullary volume of the differentiating ovary remains smaller than that of the differentiating testis. Aromatase activity starts to increase from the beginning of TSP and the estrogens produced during TSP inhibit testicular cord/tube development. The ovarian cortex begins to organize itself during TSP and grows by germ-cell divisions but more slowly than testicular cords during the same period; it grows faster after TSP and is thick at the end of the embryonic life when aromatase activity is at its maximum level (stages 25–26).

DISCUSSION

The data presented here bring new insights into two aspects, intersexuality and growth, of gonadal differentiation in a reptile with temperature-dependent sex determination (TSD).

Gonadal intersexuality

It has been claimed that in reptiles with TSD, the temperature exerts an all-or-none effect on gonadal differentiation, the individuals developing as males or females but rarely as intersexes (Crews et al., '94). However, several intersexes with ovotestes have been described among immature and mature individuals of turtle species exhibiting TSD (see discussion in Pieau, '74b; reviewed in Forbes, '64 and Raynaud and Pieau, '85). In *Emys orbicularis*, gonadal intersexuality occurs during embryonic development in artificial (Pieau, '76; Raynaud and Pieau, '85; this paper) as well as in field incubations (Pieau, '74b, '82). We show here that the formation of ovotestes at the pivotal temperature is correlated with slight increases of aromatase activity, compared to that in testes, during TSP. Considering that aromatase activity reflects estrogen synthesis (Desvages et al., '93), it can be assumed that estrogen levels—lower than in ovaries but slightly higher than in typical testes during TSP—are sufficient to induce formation of an ovarian-like cortex but not sufficient to inhibit development of testicular cords. By the end of the embryonic life, aromatase activity in ovotestes becomes similar to that in typical testis. This corresponds to the beginning of degeneration of germ cells in the ovarian-like cortex of ovotestes. This process continues after hatching. In most individuals, ovotestes evolved as typical testes, showing that gonadal intersexuality is a transient phenomenon that occurs in potential males. However, in a few individuals, some oocytes may escape degeneration and are maintained at the surface of gonads, which otherwise display typical testicular tubes. In such ovotestes, the growth of oocytes is delayed compared to that in ovaries. It can therefore be assumed that these gonads also become functional testes in adults. Such a mechanism might explain the different cases of intersexuality previously described in immature and mature turtles (reviewed in Forbes, '64; Raynaud and Pieau, '85).

Previous experiments in *E. orbicularis* and other reptilian species with TSD have shown that
aromatase plays a key role in gonadal differentiation and suggested that temperature could intervene by different mechanisms in the transcriptional regulation of the aromatase gene (Pieau, ’96). On the other hand, it is clear that temperature action is superimposed on a genetic component of sex determination because both males and females are obtained at the pivotal temperature. In E. orbicularis, this genetic component would correspond to a female heterogamety (Zaborski et al., ’88). At the pivotal temperature, males exhibit various degrees of ovarian-like cortex development that are well correlated with aromatase activity, probably reflecting subtle regulations of the aromatase gene at the transcriptional level. Both genetic (unknown) and epigenetic (temperature) factors would be involved in these regulations.

Various cases of gonadal intersexuality, natural or experimentally induced, have been described in vertebrates. In nonmammalian species, as in E. orbicularis, the formation of ovo tes tes probably depend on the levels of estrogens, because these steroids have been shown to be involved in the normal process of gonadal differentiation (Pieau et al., ’94). In marsupials, ovo testes were obtained after treatment of the young in the pouch with exogenous estrogens (Burns, ’61; Shaw et al., ’88). The problem remains open for eutherian mammals in which the implication of estrogens in gonadal differentiation is not yet established (Pieau et al., ’94). However, in our opinion, it would be of particular interest to examine estrogen synthesis and aromatase expression (mRNA) in models such as pig (Hunter, ’96) or the mole Talpa occidentalis (Jiménez et al., ’93), which present, respectively, a great incidence and a general incidence of intersexuality in XX individuals.

**Gonadal growth**

Differential growth of gonads—the growth rate of XY gonads being faster than that of XX gonads—is well documented in mammals. It begins before sexual differentiation of the gonads and seems to be associated with a faster development of XY embryos (Mittwoch, ’89). Based on these observations, a model has been proposed “in which gonadal differentiation depends on developmental thresholds: the formation of Sertoli cells needs to occur by a particular stage in time in a sufficiently developed gonad, failing which the gonad will enter the ovarian pathway” (Mittwoch, ’89).

We also observe that during TSP in E. orbicu laris, gonads of embryos incubated at a male-producing temperature grow faster than gonads of embryos incubated at a female-producing temperature. Moreover, we show that this differential growth is well correlated with gonadal aromatase activity and therefore is controlled by the estrogens synthesized in the gonads. The first event that clearly distinguishes a differentiating testis from a differentiating ovary is that in a testis medullary cords develop whereas in an ovary they do not. At the same time, the germinal epithelium is maintained at the surface of the ovary; however, the important proliferation of germ cells in the so-called cortex occurs somewhat later. Both phenomena—inhibition of testicular cord development and stimulation of cortex development in differentiating ovary—also occur with the same chronology after treatment with estrogens during TSP at a male-producing temperature. This has been observed in E. orbicularis (Pieau, ’74a; Dorizzi et al., ’91) and described in detail in Trachemys scripta (Wibbels et al., ’93). However, differential growth of gonads does not always display the same pattern as the one described here. Patterns vary according to the temperatures used to yield males and females, and to species. Thus, in E. orbicularis, at 28.5°C (pivotal temperature), ovaries become clearly smaller than testes only by the end of TSP (compare Fig. 2E and 2D), whereas gonads developing at 30°C are somewhat smaller than those at 25°C as of the beginning of TSP (Fig. 4). In the sea-turtle Dermochelys coriacea, ovaries remain smaller than testes up to hatching whatever the male and female-producing temperature used. Indeed, in this species, proliferation of germ cells is limited during embryonic development and these cells have not entered meiosis at hatching (Rimblot et al., ’85). At hatching, in E. orbicularis, the ovarian cortex is thick (Pieau, ’74a ), therefore the size of the ovaries is similar or somewhat higher than that of testes.

In conclusion, intersexuality and differential growth of gonads in E. orbicularis may be simply explained by the opposite effects of endogenous estrogens on the superficial and inner parts of the gonads. Once more, these results emphasize the importance of aromatase in gonadal differentiation.

**LITERATURE CITED**


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