Sex Reversal and Aromatase in the European Pond Turtle: Treatment With Letrozole After the Thermosensitive Period for Sex Determination

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ABSTRACT In the European pond turtle (Emys orbicularis), gonadal sex differentiation is temperature-dependent. The temperature sensitive period (TSP) of gonadogenesis lies between stages 16 and 22 of embryonic development. Previous studies have shown that embryos incubated at 30°C, a temperature yielding 100% phenotypic females, can be sex reversed by treatments with an aromatase inhibitor administered during TSP or even somewhat after TSP (as of stage 22+).

The goal of the present study was to determine whether the ovary still retains male potential at later stages of embryonic development and whether the induced male characters persist after hatching. For this purpose, eggs of E. orbicularis were treated with letrozole, a nonsteroidal aromatase inhibitor, at or as of stages 23, 24 or 25, then gonadal aromatase activity in each individual and the related gonadal structure were studied at hatching (stage 26) and for one year after hatching. Two kinds of treatments were carried out: 1) repeated applications of 10 μg of letrozole in ethanolic solution onto the eggshell; and 2) a single injection of 10 μg of letrozole in olive oil. Similar results were obtained with either application or injection of the aromatase inhibitor. In treatments as of or at stage 23, individuals with gonadal aromatase activity lower than 20 fmoles/hour/gonad had ovotestes, i.e., 22% of the treated individuals. At hatching, the inner part of these ovotestes contained testicular cords and also mixed lacunae presenting various degrees of transdifferentiation of the epithelium into a Sertolian epithelium. The cortex was maintained, although some germ cells degenerated within it. These processes continued after hatching. However, at 12 months, gonads were still ovotestes displaying some follicles with a growing oocyte in the remaining parts of the cortex. In treatments as of or at stages 24 or 25, only a few individuals were masculinized. One had ovotestes; in others, the cortex was absent in some parts and when it was present oocytes were degenerating. These results show that in the European pond turtle, differentiation of ovotestes from ovaries can be induced by treatment with an aromatase inhibitor starting at late stages of embryonic development (between the end of TSP and hatching), although such differentiation is less frequent as embryonic development proceeds. Sex reversal persists for at least one year after hatching. J. Exp. Zool. 290:490–497, 2001. © 2001 Wiley-Liss, Inc.
The objective of the present study was to further characterize the male potential of embryonic ovaries by determining how long after TSP female sex reversal can be obtained, and to examine the persistence of sex reversal after hatching. For this purpose, we have carried out treatments with letrozole at three late stages of embryonic development at 30°C, between the end of TSP and hatching, when aromatase activity strongly increases in differentiating ovaries (Desvages and Pieau, '92; Pieau et al., '98). The effects of these treatments have been followed as of the end of embryonic development and up to one year after hatching, by measuring the gonadal aromatase activity and examining the related gonadal structure.

**MATERIALS AND METHODS**

The nonsteroidal aromatase inhibitor (letrozole CGS 20267) was from Ciba Geigy (Basel, Switzerland). It was administered at or as of stages 23, 24 or 25 of *E. orbicularis* embryonic development, according to two different protocols (Fig. 1) either a single injection of 10 μg of letrozole in 50 μl of olive oil (series I, II, III), or successive applications of 10 μg letrozole in 10 μl of ethanol onto the eggshell (series IV, V, VI) as described in Dorizzi et al. (’94). Depending on the starting stage, successive applications (every 3–4 days), 7 as of stage 23 (series IV), 6 as of stage 24 (series V) and 3 as of stage 25 (series VI), were made. Control embryos received the same volume of ethanol or olive oil alone.

Many animals, among which were normal 25°C control males, were reared up to one year after hatching. They hibernated during the sixth and seventh months. Their feeding activity was moderate before hibernation and intensive afterwards up to 12 months.

Aromatase activity was measured at the beginning of each experiment as a control of the levels of aromatase activity at stages 23, 24 and 25, and then at different stages up to one year after hatching. Measurements were made in one gonad of each individual, with the tritiated water technique using [1β-3H] androstenedione as substrate and results are expressed in fmoles per hour and per gonad as previously described (Dorizzi et al., ’94). The gonad was fixed after incubation for the aromatase activity assay. Given that at the end of embryonic development and 34–36 days after hatching, slightly masculinized ovaries with 20 to 30 fmoles/hour/gonad of enzyme activity had been previously obtained after letrozole treatment at stage 22+ (Dorizzi et al., ’96), the gonads with an aromatase activity lower than 30 fmoles/hour/gonad were systematically sectioned and stained for histology. In addition, one gonad of some treated individuals with higher aromatase activity and one ovary of some control females at different stages were also processed for histological examination.

Statistical analysis was performed with Statview 5.1 (Abacus Concept). Aromatase activities were log-transformed to equalize variances. Two-way ANOVA was used for the analysis of the effects of both the method and the stage of treatment, and one-way ANOVA was used for the effects of treatments (comparison between control and treated individuals). Significance levels were set to 0.05 (*), 0.01 (**) and 0.001 (***) in Table 1.

**RESULTS**

**Gonadal aromatase activity**

Gonadal aromatase activity in each individual from the different experimental series is shown in Fig. 2. Similar results were obtained with either of the protocols used for treatments (ANOVA, F(1,118) = 0.139; P = 0.7). Therefore, results obtained with both protocols have been grouped for calculation of the mean value ± SEM of the aromatase activity in control and letrozole-treated individuals of the same stage as given in Table 1.

**Treatments as of, or at, stage 23 (series I and IV)**

At stage 23, the beginning of the treatment, gonadal aromatase activity varied depending on the
individuals with a mean value ± SEM of 31.6 ± 3.5 femtomoles/hour/gonad (Table 1) and a difference of 50.6 femtomoles/hour/gonad between the maximum and the minimum values.

Treated individuals were sacrificed at stages 24 and 25 of embryonic development, at hatching and five, nine and 12 months after hatching (Fig. 2A).

At stage 24, gonadal aromatase activity remained high (> 40 femtomoles/hour/gonad) in all individuals and the mean value ± SEM of this activity was not significantly different between letrozole-treated embryos and controls. The mean value of treated individuals compared to controls was significantly lower at stage 25 and at hatching. After hatching, the difference between treated and control mean values was significant at 12 months only. However, at all stages, several or some individuals had gonadal aromatase activity lower than the lowest one among controls; 27 treated individuals out of a total of 106 sacrificed as of stage 25 were in this case. The aromatase activity of all these individuals was lower than 30 femtomoles/hour/gonad and in nine individuals among them, the aromatase activity was lower than 5 femtomoles/hour/gonad, i.e., close to that in control testes.

Treatments as of, or at, stage 24 (series II and V)

At stage 24, the beginning of the treatment, gonadal aromatase activity had increased compared to that at stage 23, with a mean value of 92.6 ± 7.3 femtomoles/hour/gonad. There was again a great range of individual values, the minimum being 23.3 femtomoles/hour/gonad, a value under the mean value of stage 23, and the maximum being 185.4 femtomoles/hour/gonad.

Treated animals were sacrificed at hatching, and three and 12 months after hatching (Fig. 2B). At hatching and three months, the mean values of gonadal aromatase activity were not significantly different between treated and control individuals. However, three individuals at hatching and one at three months displayed aromatase activity below the lowest control level and two of these individuals had very low enzyme activity. At 12 months, all values in treated individuals were above the lowest control individual, although the mean value in treated animals was significantly lower than in control animals (Table 1).

Treatments as of, or at, stage 25 (series III and VI)

At stage 25, the beginning of the treatment, the mean value of aromatase activity of 106.3 ± 8 femtomoles/hour/gonad was very close to that at stage 24 but with a minimal value of 55.2 femtomoles/hour/gonad, which is more than twice the lowest value obtained at stage 24.

Treated animals were sacrificed at hatching and five, nine and 12 months after hatching (Fig. 2C). Except at nine months, differences of the mean values of aromatase activity between control and treated animals were not significant (Table 1). However, of 38 treated animals, six had gonadal aromatase activity below the lowest level in control females, and in five of them the aromatase activity was lower than 30 femtomoles/hour/gonad.

Gonadal structure

As described previously (Pieau, ’74; Dorizzi et al., ’94, ’96), at stages 23, 24 and 25, gonads of E. orbicularis embryos incubated at 30°C presented ovaries with a developing cortex containing germ cells in meiotic prophase and a medulla composed of...
of loose mesenchymal tissue containing thin epithelial cords and lacunae lined with flat epithelial cells. Sex reversal of gonads thus implied differentiation of testicular cords from medullary ovarian structures and regression of cortex.

**Treatment as of, or at, stage 23 (series I and IV)**

The gonads of all treated individuals with a level of aromatase activity lower than 20 fmoles/hour/gonad (22% of the animals) clearly showed these processes of sex reversal even though after hatching aromatase activity in some controls was somewhat lower (Fig. 2A). Discrete signs of ovarian masculinization were still recognizable between 20 and 30 fmoles/hour/gonad but not at higher levels of enzyme activity.

In masculinized gonads at hatching, the cortex was maintained, although a few germ cells degenerated; the medulla contained testicular cords, lacunae with flat epithelium and also mixed lacunae that presented both a flat epithelium and a higher epithelium of the Sertolian type. These mixed lacunae were transdifferentiating into tes-
ticular tubes. Moreover, the medulla was infiltrated by lymphoid-like tissue that reached the cortex (Fig. 3A,B).

Differentiation of testicular cords/tubes and degeneration of the cortex continued after hatching (Fig. 3C,D,E). However, at 12 months, some testicular cords presented degenerating cells (Fig. 3D) and/or were lacunar (Fig. 3E); parts of the cortex were maintained, presenting in some places hollows left by degenerated germ cells, or enclosing in other places follicles with growing oocytes (Fig. 3C,D). Infiltrations of lymphoid-like tissue extended up to the surface of the gonads where the cortex was completely or almost completely destroyed (Fig. 3F).

**Treatment as of, or at, stage 24**
(series II and V)

At hatching, the animal with a very low gonadal aromatase activity presented oovestes as in individuals treated as of or at stage 23. At three months, in the gonad of the animal with the lowest aromatase activity (4.5 fmoles/hour/gonad), the cortex was absent in some parts and when it was present, the germ cells were degenerating; however, testicular-like cords were not found in the medulla. At 12 months, all gonads were ovaries.

**Treatment as of, or at, stage 25**
(series III and VI)

The gonads were ovaries except in the nine month-old animal with the lowest aromatase activity. In this animal, the gonadal structure was similar to that observed in the three month-old animal treated as of, or at, stage 24.

**DISCUSSION**

In *E. orbicularis* embryos incubated at 30°C (a female-producing temperature), the ovaries present at the end of TSP, as well as a cortex with germ cells entering meiotic prophase and a medulla containing thin epithelial cords and lacunae lined by a flat epithelium within a loose mesenchymal tissue. Sex reversal after TSP thus requires two main processes: degeneration of cortex, and development of seminiferous cords from medullary structures. The present study shows that these processes can be induced, at least in some individuals, by treatments with an aromatase inhibitor (letrozole) administered as of or at three stages (23, 24 and 25) between the end of TSP (stage 22) and hatching (stage 26), thus extending our previous observation (Dorizzi et al., '96) to later stages of embryonic development. However, as the embryonic development proceeds, treatments are less effective.

After treatments with letrozole at these late stages, many, but not all, germ cells degenerated in the cortex, a process that seems linked to infiltrations of lymphoid-like tissue in the medulla as previously suggested in *Chelydra serpentina* (Yntema, '81). In the medulla of these treated individuals, testicular cords differentiated from thin epithelial cords and also from ovarian lacunae. The epithelium of lacunae thickened and acquired Sertolian characteristics, as was also observed after treatments with letrozole or fadrozole before and/or during TSP or somewhat after TSP (Dorizzi et al., '94, '96). Such a transdifferentiation of ovarian lacunae into testicular cords/tubes also occurred in gonads of female chickens after treatment with fadrozole administered before gonadal sex differentiation: SF1, SOX9, and AMH genes were shown to be expressed in the thickened part but not in the flattened part of the epithelium of mixed lacunae; in males, these genes are expressed in Sertolian cells of testicular cords (Vaillant et al., 2001). These observations suggest that testicular cords/tubes and ovarian lacunae have a common origin. Accordingly, during normal gonadal differentiation in reptiles the epithelium of testicular cords and that of ovarian lacunae have been shown to derive from the same lineage, i.e., cells proliferated by the germinal epithelium (Pieau et al., '99a).

In *E. orbicularis*, treatments with aromatase inhibitors carried out before and/or during TSP at 30°C resulted in differentiation of oovestes and...
also of almost typical testes at hatching (Dorizzi et al., '94), whereas the responses to treatments performed after TSP depended on the level of aromatase activity at the beginning of treatments, and never resulted in typical testes (Dorizzi et al., '96, and this paper). Even in the most masculinized gonads obtained from treatments after TSP, the ovarian cortex was still present at hatching and after hatching; thus, only ovotestes were obtained. We show here that one year after hatching, these gonads contain testicular cords together with follicles containing large oocytes.

Ovotestes were also obtained by incubating eggs of E. orbicularis at the pivotal temperature of 28.5°C (Pieau et al., '98). However, in the case of treatments with letrozole after TSP, the development of ovotestes was delayed; at hatching, it was similar to that at stage 23 when incubated at 28.5°C. This shows that transdifferentiation proceeds more slowly than normal differentiation.

After hatching, the cortex of ovotestes generally regressed in individuals hatched from eggs incubated at 28.5°C, but in some individuals parts of this cortex were maintained and ovarian-like follicles developed in them (Pieau et al., '98). In individuals treated with the aromatase inhibitor after TSP, parts of the cortex with developing follicles were maintained at least up to one year (this paper). Nevertheless, it can be assumed that these gonads are really sex reversed, since among TSD turtles captured in nature, several animals presented ovotestes with fertile seminiferous tubes (producing spermatozoa) and some immature oocytes; these animals were considered as functional males (reviewed by Pieau et al., '99b).

Our results for aromatase activity show different responses to treatments with letrozole, not only depending on the stage of treatment, but also depending on the individuals in each experimental series. This variability can reflect the variability of levels of gonadal aromatase activity between individuals at the time of treatment. The mean value of gonadal aromatase activity was more significantly lowered after treatment at or as of stage 23, in particular at hatching. Twenty-two percent of treated animals had ovotestes in treatments performed or beginning at stage 23, whereas in later treatments only one animal had ovotestes, and in a few individuals, only some cortex degenerations were observed. Masculinization may have occurred in animals presenting the lowest values of gonadal aromatase activity at the beginning of treatment.

In control ovaries, aromatase activity decreased after hatching and up to hibernation. It increased again after hibernation, when folliculogenesis takes place. This aromatase activity profile could correspond to a change in the site of aromatase synthesis and activity, i.e., in the medulla during embryonic development, as shown by detection of aromatase mRNA in situ hybridization in the chick embryos (Yoshida et al., '96), and in follicular cells after hatching. The inhibition of estrogen synthesis with letrozole does not mean the disappearance of the estrogens already present in the gonad at the time of treatment, and these hormones could thus continue at least for a certain (unknown) time to exert their feminizing effects on the gonads (Desvages and Pieau, '92). Furthermore, related to the estrogen levels, the degree of differentiation of ovarian components, in particular that of lacunar epithelium, was more advanced at stages 24 and 25 than at stage 23, perhaps explaining the greater difficulty encountered in observing sex reversed gonadal phenotypes at later stages.

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LITERATURE CITED


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