

Sensitive Stages for the Effects of Temperature on Gonadal Aromatase Activity in Embryos of the Marine Turtle *Dermochelys coriacea*

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Many data suggest an involvement of estrogens in gonadal differentiation in reptiles with temperature-dependent sex determination (TSD). However, the site of estrogen synthesis in two species of freshwater turtles is unclear. In *Emys orbicularis*, estrogens were shown to be produced by the gonads, whereas in *Trachemys scripta*, gonadal steroids were not detected. The marine turtle *Dermochelys coriacea* exhibits TSD but in gonadal development, ovarian differentiation is delayed. Gonadal aromatase activity and estrogen content in this species were measured in embryos incubated at 27° and in embryos incubated at 30.5°, respectively, masculinizing and feminizing temperatures within the range of temperatures found in natural nests. At all stages studied, aromatase activity was present and found to be higher at 30.5° than at 27°. Estrogens were only found at 30.5°. The effects of temperature shifts on gonadal aromatase activity were then examined. Eggs were shifted from 27 to 35° (feminizing temperature) at different embryonic stages and exposed to 35° for 6 days. An increase in gonadal aromatase activity, although with significant individual variations, was seen only when eggs were shifted between stages 23 and 27. These stages are in the range of the thermosensitive stages for sexual differentiation of the gonads determined in other turtles. These results are similar to those previously obtained in *E. orbicularis* and agree with a key role for endogenous estrogens in gonadal differentiation of reptiles with TSD. Relatively high levels of these hormones are probably required to inhibit the development of testicular cords in all species, but the sensitivity to estrogens of the surface epithelium of the gonads varies with species. © 1993 Academic Press, Inc.

Estrogens are known to be involved in avian and reptilian gonadal differentiation. In birds, which display a male ZZ/female ZW system of genotypic sex determination, injection of estrogens into eggs induces partial or complete sex-reversal of the gonads in genotypic males, whereas injection of tamoxifen (an antiestrogen) results in total masculinization of the right gonad and partial masculinization of the left gonad in genotypic females (see Scheib, 1983). Genotypic female chickens can be sex-reversed by early treatment of embryos with aromatase inhibitors (Elbrecht and Smith, 1992; Wartenberg *et al.*, 1992). In reptiles, the feminization of gonads due to the effects of exogenous estrogens have been obtained in genotypic males of species with

genotypic sex determination (Raynaud, 1967) and at a male-producing temperature in species with temperature-dependent sex determination (TSD) (Pieau, 1974; Bull *et al.*, 1988). Moreover, in the European pond turtle (*Emys orbicularis*), a species displaying TSD, the injection of tamoxifen into eggs incubated at a feminizing temperature results in differentiation of testicular cords in the gonads (Dorizzi *et al.*, 1991).

Steroid hormones are synthesized very early in avian gonadal development. For example, in quail embryos, estrogen content as well as aromatizing capacities are much higher in gonads of genotypic females than in genotypic males before any morphological indication of sexual differentiation (Scheib *et al.*, 1985). Steroid synthesis

and aromatase activity in the gonads have also been studied in the European pond turtle in which embryonic gonads metabolize different radiolabeled steroid precursors (pregnenolone, progesterone, dehydroepiandrosterone, androstenedione) during and after the thermosensitive period of sexual differentiation (Desvages and Pieau, 1991). During the early stages of this period, gonadal estrogen content is higher in embryos incubated at a feminizing temperature than in embryos incubated at a masculinizing temperature (Dorizzi *et al.*, 1991). Aromatase activity remains very low in differentiating testes from the beginning of the thermosensitive period to hatching. In differentiating ovaries, the enzyme activity increases in an exponential fashion up to the end of embryonic life and decreases around hatching. Moreover, a shift of several days from a masculinizing to a feminizing temperature during the thermosensitive period for sexual differentiation produces an increase in gonadal aromatase activity, but is ineffective after this period (Desvages and Pieau, 1992a). The sensitive period for the effects of temperature on aromatase activity in the gonads may thus correspond to that for their sexual differentiation.

In embryos of another emydid turtle, *Trachemys scripta*, there is active steroidogenesis in the mesonephros and adrenals (not dissociated) but steroids (progesterone, testosterone, estradiol, and corticosterone) were not detected in gonads (White and Thomas, 1992). Moreover, radiolabeled 17β -estradiol bound to the mesonephros, Müllerian ducts, and interrenals, but few if any estrogen-binding cells were found in the gonads during and after the temperature-sensitive period for sex determination (Gahr *et al.*, 1992). These suggest that estrogenic actions on gonadal differentiation may be indirect.

Given the apparent discrepancy between *E. orbicularis* and *T. scripta*, it was of interest to investigate the estrogen content and aromatase activity as a function of temperature in a turtle from a different family.

The marine turtle *Dermochelys coriacea* (family Dermochelyidae) also exhibits TSD. Both phenotypic males and phenotypic females can be obtained around 29.5° (pivotal temperature). Lower temperatures produce 100% phenotypic males, whereas higher temperatures give 100% phenotypic females (Rimblot *et al.*, 1985; Rimblot-Baly *et al.*, 1986–1987). By comparison with other marine and freshwater turtles, the sexual differentiation of ovaries appears to be delayed. At hatching, the gonads of phenotypic females present a very reduced medullary part with thin epithelial cords and lacunae. The surface epithelium is thick, pseudostratified, and encloses germ cells. However, the latter are not numerous and have not yet entered meiotic prophase. For these reasons, the gonads in hatchling phenotypic females of *D. coriacea* have been considered as “potential” ovaries (Rimblot *et al.*, 1985). In hatchlings of other turtle species, such as *Chelonia mydas* (Miller and Limpus, 1980), *Caretta caretta* (Yntema and Mrosovsky, 1980), *E. orbicularis* (Pieau, 1974), and *T. scripta* (Wibbels *et al.*, 1991), epithelial cords are no longer recognizable or are very reduced in the inner part of the ovaries; in the cortex, germ cells are numerous, many of them have entered meiosis and primary follicles are formed.

The present study assesses first whether the aromatase activity is present and estrogens are synthesized in embryonic gonads of *D. coriacea*, and second whether the delay of gonadal differentiation in *D. coriacea* can be correlated with a delay of the sensitive period for the effects of temperature on gonadal aromatase activity. Gonadal aromatase activity and estrogen content have been compared at a male-producing temperature and at a female-producing temperature both in the range of temperatures encountered naturally. The sensitive period for aromatase activity has been determined by studying the response to shifts from a masculinizing to a highly feminizing temperature at different embryonic stages.

MATERIALS AND METHODS

Collection and Incubation of Eggs and Staging of Embryos

Eggs of *D. coriacea* were collected on a beach (Ya:lima:po) in French Guiana. Samples of 27 to 83 eggs from seven different clutches were taken immediately after being laid, put into styrofoam boxes, and transported to Paris. In the laboratory, eggs were placed in one layer in styrofoam boxes with moist sand as substrate and incubated either at 27° (masculinizing temperature) or at 30.5° (feminizing temperature) (Rimblot *et al.*, 1985). Both temperatures are in the range of temperatures existing in natural nests.

To determine the stages at which an increase in incubation temperature results in an increase in gonadal aromatase activity, eggs were first incubated at 27° until different embryonic stages and then shifted to 35° for 6 days. Thirty-five degrees Celsius was chosen as the feminizing temperature, since it has been shown that in emydid turtles, which display the same pattern of response to temperature as *D. coriacea*, the higher the feminizing temperatures, the greater the female potencies (Bull *et al.*, 1990) and the stronger the feminization of gonads (Pieau, 1978). Moreover, shifts from 25 to 35° give a clear response in aromatase activity in embryos of *E. orbicularis* (Desvages and Pieau, 1992a,b). A total of 82 shifted eggs and 82 control eggs (maintained at 27°) of *D. coriacea* were used in these experiments.

At each time a series of eggs was shifted, one embryo was weighed and fixed *in toto* in Bouin's solution for staging. All embryos taken for the assays were weighed, and the head, the carapace, and the limbs of some of them were fixed for staging, after removal of gonads. Staging was based on weight and morphological criteria described for the marine turtles (Miller, 1985; Renous *et al.*, 1989). In many studies, staging of turtle embryos is referred to developmental stages described in *Chelydra serpentina*, a freshwater turtle (Yntema, 1968), although designation of stages in this turtle is different from that in marine turtles. Table 1 gives the age at 27°, the age at 30.5°, the weight of embryos from stages 22 to 31 (hatching) in *D. coriacea*, and the corresponding stages in *C. serpentina*.

Measurement of Gonadal Aromatase Activity

The assays were carried out between stages 23 and 31, each of them on the two gonads of an individual. The gonads were removed and were either kept *in toto* up to stage 28 or cut into four pieces by a longitudinal and a middle transverse section at stages 29 to 31. They were stored briefly in cold medium (RPMI 1640, Hepes buffer) and incubated for 4.5 hr at 36° in 0.4 ml RPMI containing 0.5 μ M [1β - 3 H]androstenedione (27.5 Ci/mmol, New England Nuclear) as substrate. At

TABLE 1
DEVELOPMENTAL STAGES AS A FUNCTION OF AGE AND WEIGHT IN EMBRYOS OF *D. CORIACEA* AND CORRESPONDING STAGES IN *C. SERPENTINA* (YNTEMA, 1968)

<i>D. coriacea</i>				<i>C. serpentina</i>
Age at 27° (days)	Age at 30.5° (days)	Weight (g)	Stages	Stages
30-33	19-21	0.3-0.5	22	15
33-36	21-23	0.5-0.8	23	16
36-39	23-25	0.8-1.4	24	17 and 18
39-43	25-28	1.4-3	25	19 and 20
43-48	28-32	3-6	26	21
48-54	32-37	6-13	27	22 and 23
54-61	37-44	13-21	28	24
61-70	44-50	21-32	29	24
70-76	50-54	32-43	30	25
76-78	55-57	38-48	31	26

the end of the incubation, radioactivity was measured in the medium after steroid extraction by chloroform followed by dextran-charcoal adsorption. Aromatase activity was calculated from the rate of [3 H]water released from the 1β -position of the substrate (Ackerman *et al.*, 1981). In results, activity is expressed in fmol per gonad per hour.

For statistical analysis, a log transformation of results was used to equalize variances. When comparing three treatment groups at the same embryonic stage, Bartlett's test was used to test the inequality of variances. If variances were not significantly different ($P < 0.05$), ANOVA tests were used to estimate the residual variance, then results in the different treatment groups were compared with the two-tailed *t* test. If variances were different, results were compared with the Mann-Whitney test.

Measurement of Gonadal Estrogen Content

Gonadal estrogen content was measured at stages 26 and 31 (hatching) in individuals incubated at 30.5°. The assays were carried out on pools of 22 gonads at stage 26 and 8 gonads at stage 31. After homogenization of the gonads, steroids were extracted with 0.5 ml diethylether, repeated three times. Extracts were evaporated to dryness in a stream of nitrogen, dissolved in 130 μ l of 50 mM Tris-HCl, pH 7.4, and centrifuged at 10,000g for 5 min. Estrone and estradiol concentrations were measured according to Nicolas *et al.* (1979) using the transhydrogenase function of 17β -estradiol dehydrogenase of human placenta. This method involves an NADPH regenerating system, by which NADH accumulates during the enzymatic cycling reaction. The amount of NADH produced is directly proportional to the concentration of the two estrogens. Reagents (kit Estradiol enzymatique "U," ref. 61483) were purchased from bioMérieux (France). Incuba-

tions were carried out in a final volume of 300 μ l at 25° for 22 hr. Determinations of NADH concentrations were made by spectrophotometry. Under these conditions, the rate of appearance of NADH was a linear function of estradiol concentrations between 0 and 80 pg. Twenty picograms of estradiol led to an increase of about 0.1 unit in absorbance at 340 nm. Results are expressed in picograms of estradiol per gonad.

RESULTS

Gonadal Aromatase Activity and Estrogen Content in Embryos Incubated at 30.5° or at 27°

Gonadal aromatase activity was measured, individual by individual, in embryos at stages 25, 26, and 27, just before hatching (stage 30) and at hatching (stage 31). Results, grouped for each stage, are shown in Fig. 1 and the number of individuals processed at each stage is given in Table 2.

Relatively large individual variations were seen at 30.5°. However, as early as stage 25, gonadal aromatase activity is sig-

nificantly higher at 30.5° than at 27° ($t = 2.28$, $df = 24$, $P < 0.05$). During the following stages (26 and 27), aromatase activity is still much higher at 30.5° than at 27° ($t = 4.62$, $df = 22$, $P < 0.001$ and $t = 5.67$, $df = 24$, $P < 0.001$, respectively). At 30.5° it continues to increase to peak at stage 30 ($\epsilon_U = 3.25$, $P < 0.01$). Around hatching, it appears to slightly decrease.

Endogenous gonadal estrogens were assayed at stage 26 and at hatching (stage 31) in individuals incubated at 27 and 30.5°. In none of the assays (2 at stage 26, 1 at hatching) performed at 27° were estrogens detectable. At 30.5°, gonadal estrogen contents were 11.76 and 12.47 pg/gonad for two assays at stage 26 and 37.73 pg/gonad for one assay at hatching (Fig. 1).

Effects of Shifts from 27 to 35° on Gonadal Aromatase Activity

A series of six to eight eggs was shifted from 27° to 35° at different embryonic stages, between stages 22 and 29, and then exposed for 6 days at 35°. Since the rate of embryonic development is accelerated at 35°, a number of control embryos (maintained at 27°) equal to half that of shifted embryos was assayed for aromatase activity at the same time as the shifted embryos, the other half was assayed 2 to 3 days later when they had reached approximately the same developmental stage as the shifted embryos. Results of all experimental series are grouped in Fig. 2. In Fig. 2A, aromatase activity per gonad and per hour is given for each individual as a function of its weight. In Fig. 2B, the mean aromatase activity is given for several individuals grouped according to their developmental stage (Table 1). The number of individuals processed at each stage is indicated in Table 2.

Figure 2A shows important individual variations for gonadal aromatase activity in control embryos maintained at 27° as well as in embryos shifted to 35°. However, it is clear that between 2 and 15 g, aromatase

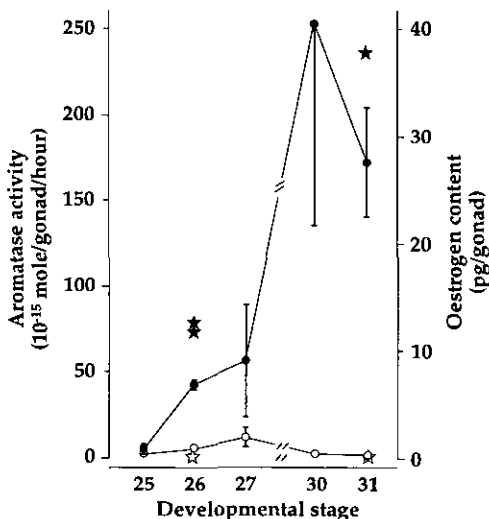


FIG. 1. Gonadal aromatase activity (● ○) and estrogen content (★ ☆) in *D. coriacea* embryos incubated at 30.5° (● ★) compared to that in embryos incubated at 27° (○ ☆). For aromatase activity, each point represents the mean value \pm SD of several individual measurements, the number of which is indicated in Table 2. Estrogen content was measured on pools of 22 gonads at stage 26 and 8 gonads at stage 31.

TABLE 2
NUMBER OF EMBRYOS AT DIFFERENT STAGES (23 TO 30) AND HATCHLINGS (STAGE 31) OF *D. CORIACEA*
PROCESSED FOR MEASUREMENT OF GONADAL AROMATASE ACTIVITY

Incubation temperature	Number of individuals at stage									Total number of individuals
	23	24	25	26	27	28	29	30	31	
30.5°	0	0	6	5	6	0	0	10	6	33
27°	5	4	9	9	15	17	8	6	9	82
27 → 35°	0	6	12	13	7	28	9	7	0	82

Note. Incubations at 27° and 30.5° correspond to data shown in Fig. 1. Incubations at 27° and 27 → 35° correspond to data shown in Fig. 2B.

activity in all 35°-incubated embryos is higher than in the 27° controls. In embryos weighing more than 21 g, aromatase activities in shifted and in control embryos were similar.

Figure 2B shows that at stage 24 (embryos shifted to 35° at stage 22), gonadal aromatase activity is not significantly different from that in control embryos incubated at 27° ($t = 1.95$, $df = 8$, $P > 0.05$). At stage 25 (shift at stage 23), a significant increase in aromatase activity is observed in shifted embryos ($t = 3.26$, $df = 24$, $P < 0.01$). At stages 26, 27, and 28 (shifts between stages 24 and 27), a quite clear response is obtained ($t = 7.21$, $df = 22$, $P < 0.001$; $t = 9.57$, $df = 24$, $P < 0.001$; $t = 5.32$, $df = 43$, $P < 0.001$, respectively), with a maximum at stage 27. An increased gonadal aromatase activity is no longer present. At stage 29, results are not significantly different in shifted and in control embryos ($t = 0.12$, $df = 15$, $P > 0.05$). At stage 30, aromatase activity is even lower in embryos shifted to 35° than in embryos maintained at 27° ($t = 2.9$, $df = 11$, $P < 0.01$). This difference is not due to a difference in embryonic weight ($t = 1.11$, $df = 11$, $P > 0.2$).

With respect to the stages at which the shifts of temperature were performed, an increased gonadal aromatase activity was only obtained when eggs were shifted to 35° within stages 23–27. Thus, in present conditions, using a very high feminizing temperature to obtain a clear response, stages

23 to 27 are the sensitive stages for the effects of temperature on gonadal aromatase activity in embryos of *D. coriacea*. In embryos incubated at 27° (masculinizing), gonadal aromatase activity increases slightly during the period between these stages, and then decreases up to hatching (Fig. 2B). The activity is maximum at the end of the thermosensitive period (stage 27).

DISCUSSION

The present investigation shows that in *D. coriacea* a marine turtle with temperature-dependent sex determination, aromatase activity is present and estrogens are synthesized in embryonic gonads. At a masculinizing temperature, aromatase activity remains low during embryonic development except for a slight peak around stage 27. Estrogens are probably produced but in too low amounts for detection. At a feminizing temperature, the aromatase activity is low at the beginning of gonadal differentiation and increases up to the end of embryonic life then slightly decreases around hatching. Estrogens are measurable and apparently increase with the same ratio as the aromatase activity during gonadal development. These results confirm and reinforce previous data obtained in the freshwater turtle *E. orbicularis*.

The failure to detect steroids and binding sites of radiolabeled 17 β -estradiol in gonads of *T. scripta* (White and Thomas, 1992; Gahr *et al.*, 1992) does not necessarily mean that estrogens and estrogen receptors

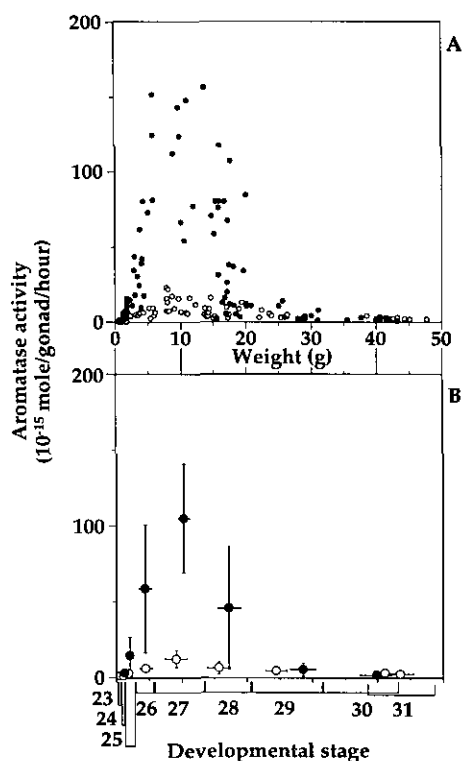


FIG. 2. Aromatase activity in gonads of *D. coriacea* embryos incubated continuously at 27° (○), or first incubated at 27° and then shifted to 35° for 6 days (●). Temperature shifts were carried out at different stages of embryonic development, between stages 22 and 29. In A, each point indicates the measured value in an individual as a function of its weight. In B, each point indicates the mean value \pm SD for several individuals grouped according to embryonic stage. Horizontal bars represent SD for the weight of pooled embryos. See Table 2 for the number of individuals processed at each stage.

are absent, rather, their levels may be below the detection limit of the assays. Thus, in *T. scripta* the detection limit was approximately 0.3 pg/gonad in the estradiol assay performed at stage 21 (White and Thomas, 1992). In embryos of *E. orbicularis* at stages 16 to 18, the estradiol content at a feminizing temperature was 0.27 pg/gonad (SD 0.11) (Dorizzi *et al.*, 1991). The higher amounts of estrogens found in *D. coriacea* can be easily explained by the size of the gonads which are much larger than in *T. scripta* and *E. orbicularis*.

In *D. coriacea*, a 6-day exposure to a highly feminizing temperature following incubation at a masculinizing temperature increases gonadal aromatase activity only during a limited period of the embryonic development. This period lies within stages 23–27 of the developmental stages defined in the marine turtles (Miller, 1985; Renous *et al.*, 1989). These stages correspond to stages 16 and 22–23 in the freshwater turtle *C. serpentina* (Yntema, 1968; Table 1). Based on specific developmental stages, experiments with temporary shifts of temperature, either upward or downward from the pivotal temperature, localized the thermosensitive period of sex determination between stages 12 and 22 in another marine turtle, *C. caretta* (Yntema and Mrosovsky, 1982). In three species of Emydidae, *Emys orbicularis* (Pieau and Dorizzi, 1981), *Graptemys ouachitensis*, and *Chrysemys picta* (Bull and Vogt, 1981), the thermosensitive period of sex determination was between stages 16 and 22. This period appears to be similar to those found in the lizard *Eublepharis macularius* (Bull, 1987) and alligator *Alligator mississippiensis* (Ferguson, 1985; Ferguson and Joanen, 1983). Comparison with these data shows that the sensitive stages for the effects of temperature on gonadal aromatase activity in *D. coriacea* are in the range of temperature-sensitive stages (or period) for sexual differentiation of gonads determined in reptiles with TSD. They agree with previous experiments in *E. orbicularis* in which a temperature-induced increase in aromatase activity was obtained during, but not after this period (Desvages and Pieau, 1992a). Thus, the thermosensitive period for gonadal aromatase activity appears to coincide with that for gonadal sexual differentiation in reptiles. The initial hypothesis that in *D. coriacea*, this period could be delayed, given the structure of ovaries at hatching is not supported.

The present study confirms that aromatase activity is correlated with estrogen

content in the gonads. In *E. orbicularis* embryos, estrogens have two effects on gonadal differentiation. First, they inhibit the development of testicular cords. Second, they induce the differentiation of an ovarian cortex which involves multiplication of germ cells and their entrance into meiosis (Dorizzi *et al.*, 1991). High levels of estrogens appear to be required for inhibition of testicular cords, while much lower levels of these hormones appear to stimulate cortical differentiation (Desvages, G., Girondot, M., Richard-Mercier, N., and Pieau, C., unpublished results). In *D. coriacea*, at feminizing temperatures, the development of testicular cords is also inhibited. However, the development of an ovarian cortex is not stimulated during embryonic life, although the levels of estrogens are relatively high. In another marine turtle exhibiting TSD, *Lepidochelys olivacea*, oogonia had not yet entered meiosis 84 days after hatching in individuals obtained from eggs incubated at 32° (feminizing temperature) (Merchant-Larios *et al.*, 1989), whereas in phenotypic females of *C. mydas* (Miller and Limpus, 1980) and *C. caretta* (Yntema and Mrosovsky, 1980), oogonia had entered meiosis at hatching. Therefore, specific responses to estrogens appear to exist for the differentiation of an ovarian cortex and probably also for the inhibition of testicular cords in turtles. These differences could account for the fact that intersexes with ovotestes are easily obtained in some species, such as *E. orbicularis* (Pieau and Dorizzi, 1981) but not in others, such as *T. scripta* (Wibbels *et al.*, 1991).

In conclusion, the changes in aromatase activity and estrogen content of embryonic gonads of *D. coriacea* are very similar to those seen in *E. orbicularis* (Dorizzi *et al.*, 1991; Desvages and Pieau, 1992a,b), although the development of gonads, chiefly ovaries, displays specific differences. A key role for the regulation of expression of the P-450 arom gene in the sexual differentiation of gonads is indicated. Further in-

vestigations in reptiles with TSD will be needed to determine both the factors regulating transcription of the P-450 arom gene (one of these factors would be thermosensitive), and the genes regulated by the produced estrogens (Pieau *et al.*, 1993).

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