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Response of Diencephalon But Not the Gonad to Female-Promoting Temperature With Elevated Estradiol Levels in the Sea Turtle *Lepidochelys olivacea*

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ABSTRACT Although temperature sex determination is well known in several reptile species, the physiological mechanism underlying this process remains to be elucidated. In the current work, we analyzed the levels of testosterone (T) and estradiol (E2) in the gonads; two brain regions—telencephalon (Te) and diencephalon/mesencephalon (Di)—and the serum of developing embryos of the olive ridley *Lepidochelys olivacea* incubated at male- or female-promoting temperatures. Conversion of pregnenolone (P5) to T and T to E2 were studied in the gonads and brain. The analyses were performed during three periods: the thermosensitive period (TSP), histologically undifferentiated gonads (UDG), and differentiated gonads (DG). In the gonads, serum, and brain, T concentrations were higher at the female-promoting temperature during the three periods, whereas in the gonads and serum, E2 levels were similar at the female and male-promoting temperature. In Di, the concentration of E2 was significantly higher at the female-promoting temperature. Biotransformation of P5 to T in gonadal tissues were slightly higher at the female-promoting temperature in TSP and increased during UDG and DG. Conversion of T to E2, however, was similar at the two temperatures during the three periods. In the brain, the Di showed a higher efficiency for transforming T to E2 at the female-promoting temperature. Our present results do not allow us to decide whether the diencephalon is the cause or the effect, but they conclusively demonstrate that, in *L. olivacea,* this region of the brain senses temperature during sex determination. *J. Exp. Zool. 280:304–313, 1998.*

Incubation temperature is well known to be sex determinant in various reptile species (Bull, '80; Janzen and Paukstis, '91). By analogy to mammals (Jost, '47) sex differentiation in thermodependent species is thought to begin in the undifferentiated gonad, where a chain of events leading to the differentiation of ovaries or testes takes place. In eutherian mammalian species, the *Sry* gene is the testis-determining factor first expressed in the undifferentiated gonad (Koopman et al., '91). In nonmammalian species, however, the role of the *Sry* gene in sex determination is not clear (Tiersch et al., '91; Coriat et al., '94; Spotila et al., '94).

Efforts to elucidate the molecular mechanisms of sex determination and differentiation in temperature-sex-determined (TSD) reptiles have focused on steroid hormones. Raynaud and Pieau ('85) were the first to suggest the key role of estrogen synthesis in sex determination and gonad differentiation. In a review of their work with the freshwater turtle *Emys orbicularis,* Pieau et al. ('94a) postulated that temperature could act directly or indirectly on the regulation of aromatase

Abbreviations used: DG, differentiated gonad; Di, diencephalon/mesencephalon; E2, estradiol; Go, gonads; P5, pregnenolone; RIA, radioimmunoanalysis; Ser, serum; T, testosterone; Te, telencephalon; TSD, temperature sex determined; TSP, thermosensitive period; UDG, undifferentiated gonad.

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gene expression in the gonad. Contrary to data from *E. orbicularis* (Dorizzi et al., '91), male embryos of *Trachemys scripta,* another TSD turtle, secreted higher estradiol (E2) levels than females (White and Thomas, '92a). Therefore, it seems that, in some species, there is no direct correlation between higher E2 in the gonad at female-promoting temperature and sex determination.

We have proposed that the neuroendocrine system may be important for sex determination in the sea turtle *Lepidochelys olivacea*. In a high-resolution study of gonad development in *L. olivacea*, we found that the undifferentiated gonad showed nerve terminals, which are not present in mammals (Merchant-Larios et al., '89). Furthermore, we found that, when isolated undifferentiated gonads were cultured, they differentiated according to the temperature at which the donor organism was incubated. The contralateral gonad did not respond to the shifted temperature (Merchant-Larios and Villalpando-Fierro, '90). Although in these experiments, only embryos at female-promoting temperature were at the TSP (recently determined; Merchant-Larios et al., '97), our general conclusion remains valid. Moreover, in a preliminary study of steroid hormone contents in gonads of *L. olivacea*, no significant differences in E2 were found in embryos incubated in male- or female-promoting temperatures (Salame-Méndez, '92). Finally, White and Thomas ('92b,c) found that adrenal-kidney complexes responded sex-specifically to gonadotropins in embryos of *T. scripta.*

In *Alligator mississipiensis,* the thermosensitive period occurs in embryos during the genital ridge formation stages, and sex is determined well before the histological differentiation of the gonads (Ferguson and Joanen, '83). In *L. olivacea*, we found that, at the male-promoting temperature, sex determination also occurs before histological differentiation of the gonad (Merchant-Larios et al., '97). Although irreversible molecular events controlled by temperature in genital ridges cannot be discounted, primary sex determination (sexual fate) may occur in a thermosensitive extragonadal organ. If primary sex determination occurs at the gonadal level, it must be assumed that thermoreceptors emit signals for the establishment of irreversible molecular processes that will lead to morphological differentiation of the gonad. If thermoreceptors were present in the brain, however, primary sex determination might take place in this organ, and differentiation of the gonad would then be a secondary event.

In the current study, we postulate the following working hypothesis: Since exogenous E2 counteracts the effect of the male-promoting temperature, at the female-promoting temperature, a higher concentration of E2 may be detected in the sensor organ during the thermosensitive period. To verify this hypothesis, we performed two experiments. The first experiment was a high-sensitivity radioimmunoanalysis (RIA) performed to measure concentrations of testosterone (T) and estradiol (E2) in the gonads, serum, and two brain regions (telencephalon, Te, and diencephalon/mesencephalon, Di). The second experiment was a biotransformation of steroid hormones in gonads and brains of *L. olivacea* during three periods of development: (1) during the TSP; (2) after TSP but before morphological differentiation of the gonad (UDG); and (3) after the gonads can be histologically differentiated (DG).

MATERIALS AND METHODS

Animals

Lepidochelys olivacea eggs were obtained from La Escobilla beach (96° 27′16″W, 15° 40′36″N), Oaxaca, Mexico, on the night they were laid, and transported to Mexico City within 12 h by ground transportation. This study used eggs from five clutches obtained between 1993 and 1995. After arrival, eggs were placed in covered plastic trays filled with moistened vermiculite. Containers of eggs from the same clutch were placed in incubators at either 27 ± 0.5°C (male-promoting temperature) or 32 ± 0.1°C (female-promoting temperature) for different experiments.

Experimental design for steroid determination

Three distinct periods during embryogenesis were sampled. The first period corresponds to the thermosensitive period (TSP) when the embryos responded to the altered temperature (Merchant-Larios et al., '97). The second period was when the gonads were still undifferentiated (undifferentiated gonad or UDG), and the third was after the gonads were differentiated histologically (DG).

Evaluation of endogenous steroids

Radioimmunoassays (RIAs) were performed in gonads (Go), serum (Ser), and two brain regions, telencephalon (Te) and diencephalon/mesencephalon (Di), taken from embryos under the six experimental series shown in Table 1. A total of 432 assays were performed.

TABLE 1. Radioimmunoassays of different embryonic tissues of Lepidochelys olivacea *incubated at male- or female-promoting temperatures*

Period[1]	Temp. (°C)	Days	Stage	Go	Te	Di	Se
TSP	27 ± 0.5	20	20/21	6(27)	2(18)[2]	2(18)[2]	6(54)[2]
TSP	32 ± 0.1	20	23/24	2(9)	2(18)	2(18)	2(18)
UDG	27 ± 0.5	27	23/24	2(9)	2(18)	2(18)	2(18)
UDG	32 ± 0.1	27	26/27	2(9)	2(18)	2(18)	2(18)
DG	27 ± 0.5	45	27/28	2(9)	2(18)	2(18)	2(18)
DG	32 ± 0.1	33	27/28	2(9)	2(18)	2(18)	2(18)

[1]TSP, thermosensitive period; UDG, undifferentiated gonad; DG, differentiated gonad; Go, gonads; Te, telencephalon; Di, diencephalon/mesencephalon; Se, serum.
[2]Number of tissues pooled. We used three pools by assay and each assay was repeated three times. Number in parentheses indicates total number of embryos.

Radiolabeled steroids

[7-3H] Pregnenolone (sp. act. 27 Ci/mmol), [2,4,6,7,16,17-3H] testosterone (sp. act. 139 Ci/mmol), and [2,4,6,7,16,17-3H] estradiol (sp. act. 140 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and purified before use by thin-layer chromatography with toluene: ethyl acetate (2:1, v/v).

Unlabeled steroids and solvents

Steroid nomenclature according to Kime ('95) is as follows: 3β-Hydroxy-5-pregnen-20-one (pregnenolone, P5); 17β-hydroxy-4-androsten-3-one (testosterone, T); and 1,3,5(10)-estratriene-3, 17β-diol (estradiol, E2). These reference steroids were obtained from Steraloids (Pawling, NY), and the purity of each steroid was checked by TLC. All organic solvents were of analytic grade.

Steroid extractions

Pooled tissues were sonicated for 20 to 30 sec in 100 µl of Ringer solution for reptiles (New, '66) in Eppendorf tubes. Blood was centrifuged (3,000*g*/4°C/10 min) to separate serum. An aliquot of each sample was taken to determine the protein content using bovine serum albumin as standard (Bradford, '76). The remaining sample was stored at –20°C until steroid contents were determined. For steroid extraction, samples of sonicated tissues and sera were transferred to conical tubes, each with ≈1,000 cpm of labeled steroids as tracers to evaluate recovery. Then, 5 ml of diethyl ether were added to each tube and mixed in a vortex for 60 sec; each extraction was repeated twice. The aqueous phase was removed after freezing in dry ice-acetone, and the organic phase was decanted into a conical tube and evaporated until dry. Average extraction efficiency ranged from 93.5 ± 7%. Results for any given steroid and all steroid assays were corrected for recovery.

Steroid purification

Each sample extract in the conical tubes was mixed with diethyl ether:methanol (2:1, v/v) and placed on chromatoplates (20 × 20 cm) covered with 0.25 mm of silica gel and an absorption indicator of UV light of 254 nm (Merck, México, D.F. México). Two lanes of the chromatoplate were loaded with 5 µl of a standard solution of nonradioactive steroids as references. Each sexual steroid was separated using three chromatographic systems (A: benzene; B: benzene:ethyl acetate, 7:3 v/v; C: benzene:methanol, 95:5 v/v). Each chromatogram was visualized with UV (254 and 366 nm) and Oërtel reagent (sulfuric acid:ethanol, 2:1 v/v) in the corresponding zones of the spots for specific reference steroids. Each sample area with a single spot in the appropriate position together with its respective rf (running factor), was separated and eluted with 1.1 ml of diethyl ether: methanol (1:1, v/v). Two aliquots of 500 µl were obtained from each eluate; one was transferred to tubes for RIA as described below, and the other was poured into a glass vial to evalute the percentage of recovery. The solvent was evaporated under vacuum in an oven (30°C), and radioactivity was measured with a liquid-scintillation spectrometer (Beckman, LS-7000).

Radioimmunoassay (RIA)

An aliquot was quantified by specific RIA for testosterone and estradiol as previously reported (Mendieta et al., '91). Briefly, 500 µl of phosphate-buffer (0.25 M, pH 7, with sodium azide and 1% gelatin), the corresponding antiserum in an appropriate dilution, and the specific radiolabeled steroid with its respective tracer (≈5000 cpm) were added to each tube, and the tubes were incubated at 4°C overnight. Bound steroid was separated from unbound steroid by adding to each tube 500 µl of a mixture of dextran-coated charcoal (6.25:

62.5% Dextran T-70:Norit A, w/w in bidistilled water) and centrifuging (3,000*g*/4°C/15 min). The supernatant was decanted into a glass vial, and 5 ml of Instagel (Packard) was added. Radioactivity in the vial was determined in a liquid-scintillation spectrometer (Beckman, LS-7000) with a maximum efficiency of 56% for tritium. The RIA methods were validated by confirming that serial dilutions gave a linear standard curve. The intra-assay coefficient of variation for all assays were less than 4%. Antisera were raised in rabbits as previously described by Bermúdez et al. ('75), and antibodies are available with one of the authors (J.H.).

Biotransformation assay

To determine the in vitro capacity of the gonads (Go), telencephalon (Te), and diencephalon/mesencephalon (Di) to biotransform radiolabeled steroid precursors into T and E2 during TSP, UDG, and DG, three experimental series were run in triplicate, using each pair of a Go, a Te, and a Di of individual embryos at each stage and temperature. To evaluate testosterone and estradiol either [3H]P5 (0.005μCi) or [3H]T (0.003μCi) respectively were diluted in 500 μl reptile Ringer (New, '66). Tissues were incubated at male- or female-promoting temperature for 1 h without cofactors. Two controls were used for each experiment: one with a non-steroidogenic tissue (forelimb) and one without tissue. At the end of the incubation period, samples were frozen and stored until evaluation of the conversion of both precursors. Methods for extraction and purification of steroids and determination of total protein concentration were the same as described for the RIA. The percentage biotransformation to T and E2 was calculated for each incubation.

Statistical analyses

To determine significant differences in steroid contents and metabolism between embryos incubated at male- or female-promoting temperatures, all data were subjected to two-way analysis of variance, as well as to Student's *t*-tests, using the Statistical Analysis System package (SAS Institute, '85).

RESULTS

Steroid contents in gonads, brain, and serum

Figure 1 shows T and E2 concentration in gonads detected at TSP, UDG, and DG. During the three periods, the T content was significantly higher in gonads from embryos incubated at female-promoting temperature than in gonads from embryos incubated at male-promoting temperature. However, no significant differences were found in E2 contents between the gonads of embryos incubated at the different temperatures.

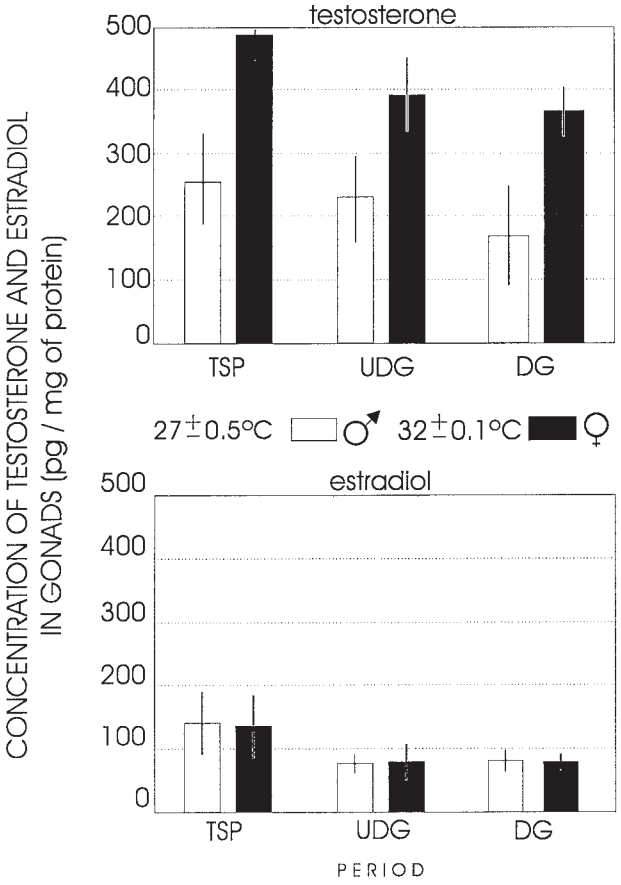

Fig. 1. Testosterone (T) and estradiol (E2) concentration in gonads of *Lepidochelys olivacea*. The differences in concentration of T between male-promoting (27 ± 0.5°C) and female-promoting (32 ± 0.1°C) temperatures during the thermosensitive period (TSP), undifferentiated gonad (UDG), and differentiated gonad (DG) are significant ($P < 0.0001$). No differences ($P = 90$) are found in concentration of E2 at either temperature during TSP, UDG, and DG.

In the brain, the contents of both T and E2 were higher in embryos from the female-promoting temperature compared to embryos at male-promoting temperature during TSP, UDG, and DG (Fig. 2). Comparing the concentrations of T and E2 in the two regions of the brain, the diencephalon/mesencephalon (Di) had significantly higher concentrations of T and E2 than the telencephalon (Te) during the three critical periods of development analyzed.

Serum samples taken from embryos incubated at female- and male-promoting temperatures had similar concentrations of T and E2 during TSP (Fig. 3). Later, during the UDG and DG stages, T concentration was higher in serum from embryos incubated at female-promoting temperature. On the other hand, E2 concentration was similar in serum from embryos incubated at female-promoting temperature during TSP and UDG. In stages of morphologically differentiated gonads (DG), E2 concentrations were higher in the serum of embryos at the female-promoting temperature.

Steroid conversion in gonads and brains

Figures 4 and 5 show the results of steroid conversion in gonads (Go) and two brain regions (Te and Di) at three periods: TSP, UDG, and DG from embryos of *L. olivacea* incubated at male- or female-promoting temperatures.

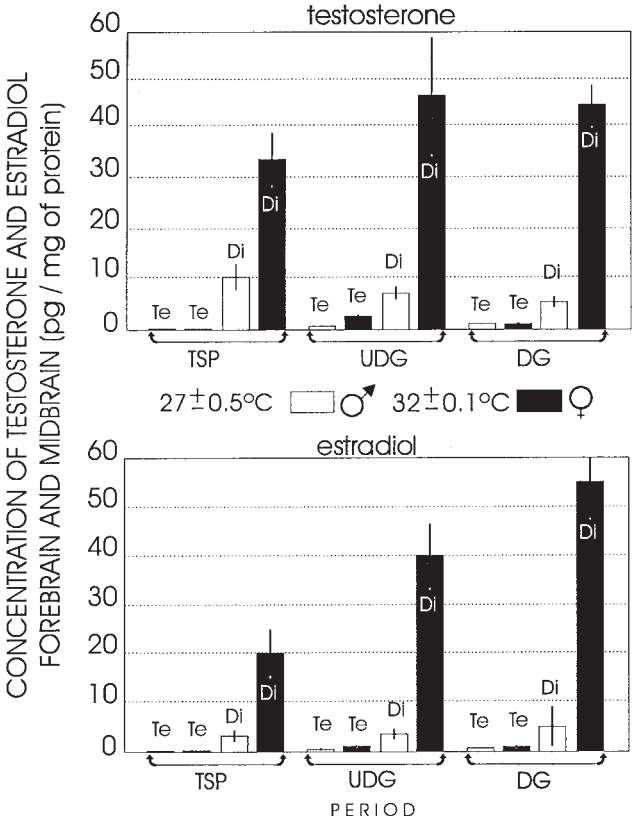

Fig. 2. Testosterone (T) and estradiol (E2) concentration in Telencephalon (Te) and Diencephalon/Mesencephalon (Di) of *Lepidochelys olivacea*. The differences in concentration of T and E2 in Di between male-promoting (27 ± 0.5°C) and female-promoting (32 ± 0.1°C) temperatures during the thermosensitive period (TSP), undifferentiated gonad (UDG), and differentiated gonad (DG) are significant ($P < 0.0001$). There are significant differences ($P < 0.001$) between Te and Di in concentration of T and E2 during the three periods at both temperatures.

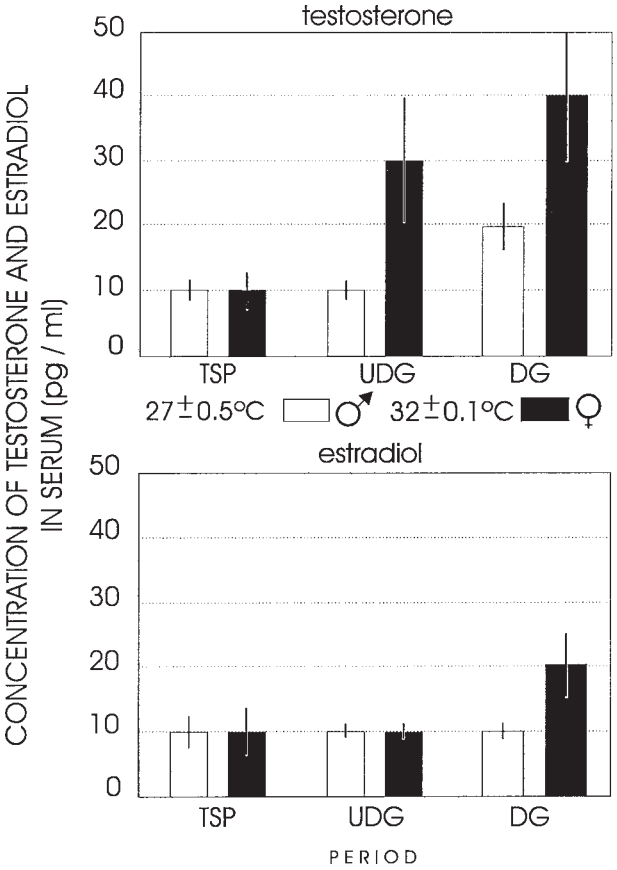

Fig. 3. Testosterone (T) and estradiol (E2) concentration in serum. The differences in concentration of T between male-promoting (27 ± 0.5°C) and female-promoting (32 ± 0.1°C) temperatures during undifferentiated gonad (UDG) and differentiated gonad (DG) are significant ($P < 0.0001$). No difference was detected ($P = 0.85$) in the thermosensitive period (TSP). The concentration of E2 is different ($P < 0.001$) only in the differentiated gonad (DG). No differences were registered ($P = 0.85$) at TSP and UDG at the two temperatures.

Biotransformation of P5 into T in Go was significantly higher at female-promoting than at male-promoting temperatures during UDG and DG. During TSP, differences were not significant (Fig. 4). In the two brain regions (Te and Di), the percentage of biotransformation of P5 into T was lower than in Go. Although Di showed a tendency to higher conversion than Te during the three periods, no significant differences between Te and Di were found (Fig. 4).

Conversion of T to E2 was higher in Go than in Te and Di, but no significant differences were found between the male-promoting and female-promoting temperatures during the three periods (Fig. 5). In the brain, percentage of T biotransfor-

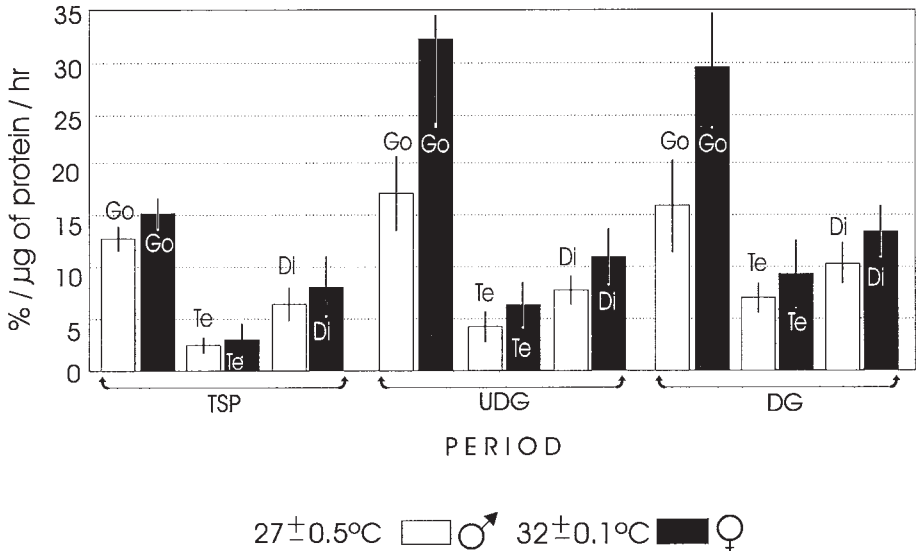

Fig. 4. Percentage of tritiated pregnenolone biotransformed into testosterone in *Lepidochelys olivacea*. Significant differences ($P < 0.001$) were present in gonads (Go) at male- or female-promoting temperatures during undifferentiated (UDG) and differentiated gonad (DG) periods. Significant differences were detected ($P = 0.85$) between Te and Di at TSP.

mation was smaller in Te than in Di. The latter tissue showed a higher tendency for transforming T to E2 at the female-promoting temperature, particularly during TSP. Thus, these results are consistent with the steroid content determinations in which the E2 concentration found in Di incubated at the female-promoting temperature was higher than that in Di incubated at the male-promoting temperature.

DISCUSSION

In the present study, three periods were used for analysis of steroid hormones in *L. olivacea*. The first was based on the thermosensitive pe-

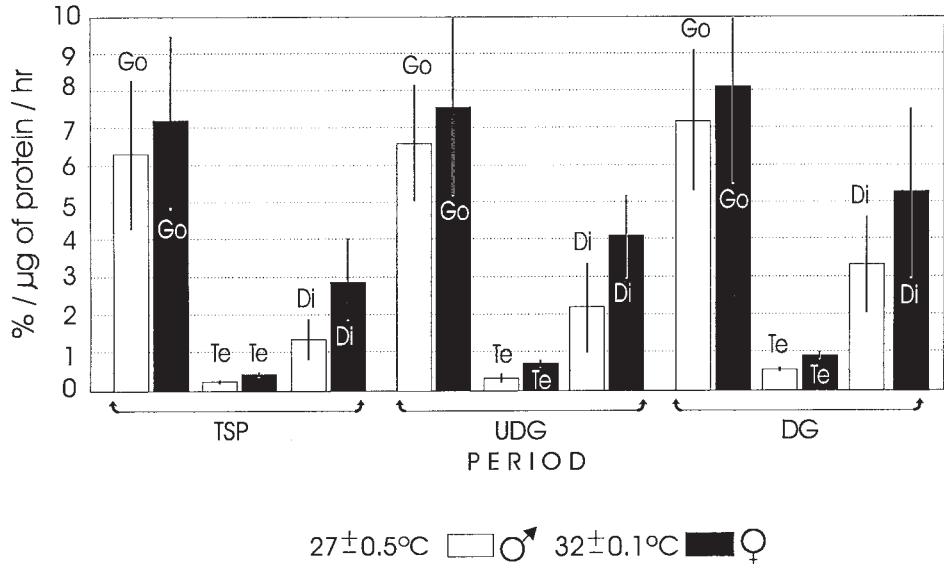

Fig. 5. Percentage of tritiated testosterone biotransformed into estradiol. Values show no significant differences in gonads (Go) ($P = 0.90$) at male-promoting (27 ± 0.5°C) or female-promoting (32 ± 0.1°C) temperatures during TSP, UDG, and DG. Te and Di show significant differences ($P = 0.80$) during the three periods. No significant differences ($P = 0.90$) are present in Di between male- and female-promoting temperatures during the thermosensitive period (TSP).

riod (TSP) (Merchant-Larios et al., '97). The other two periods referred to the histological appearance of the gonad. In the "undifferentiated gonad" (UDG) ovaries or testes cannot be distinguished and in the "differentiated gonad" (DG), ovaries and testes can be distinguished. Steroid hormone analysis was performed in TSP, UDG, and DG.

Results on the contents and production of T and E2 were interpreted as follows. During TSP, the molecular mechanism that determines the fate of gonadal sex is established. The differences in steroidogenesis at female- or male-promoting temperatures reflect a temperature-sensitive process that may be a biochemical expression of sex determination. Differences detected at both temperatures during TSP were preserved and increased during UDG and DG. This suggests that the steroidogenic pattern established during TSP is stable and precedes the histological differentiation of the gonad.

The present study of *L. olivacea* revealed significant differences in T concentrations in gonads, depending on the incubation temperature. Gonads of the embryos incubated at the female-promoting temperature produced greater amounts of T than those incubated at the male-promoting temperature. The most important difference was found at 20 days of incubation in TSP. Embryos of this age incubated at male-promoting temperature were at stages 20–21, whereas those incubated at the female-promoting temperature had reached stages 23–24. It could be argued that the detected differences depended on the size of the gonads reached at the two temperatures. However, the difference in T production was also maintained when gonads of stage 23 incubated at the female-promoting temperature were compared with gonads of similar size and histological appearance at the same stage incubated at the male-promoting temperature (see Fig. 1; Merchant-Larios et al., '97).

On the other hand, the capacity of biotransformation from P5 to T in gonads tended to be higher in embryos incubated at the female-promoting temperature during UDG and DG. In TSP however, T biosynthesis showed no differences at the two temperatures. This result contrasts with the higher concentration of T detected in gonads at female-promoting temperature during the three periods. One possible explanation is that, during TSP, the gonads of embryos incubated at the female-promoting temperature had a greater capacity to retain T.

Important differences exist between species with respect to steroid hormone metabolism and the production of estrogens. Greater activity of the P450 aromatase at the female-promoting temperature was reported during TSP in gonads of *E. orbicularis* (Dorizzi et al., '91; Desvages and Pieau, '92a,b), *Dermochelys coriacea* (Desvages et al., '93), and *Malaclemys terrapin* (Jeyasuria et al., '94). In contrast, in *Trachemys scripta,* the male-promoting temperature favours greater production of T and E2 (White and Thomas, '92a), and Lance and Bogart ('94) did not detect differences in E2 production in the urogenital ridges of *Alligator mississipiensis* incubated at female- or male-promoting temperatures during TSP. Furthermore, Smith et al. ('95) reported that higher activity of aromatase follows ovary development in the alligator. In the present study, gonads of *L. olivacea* did not reveal significant differences between the two incubation temperatures either in estradiol content or in its conversion from T. Although T content was higher at the female-promoting temperature, aromatase activity was similar at both temperatures.

No consistent gonadal development patterns exist among reptiles with TSD (reviewed by Smith and Joss, '94). Embryonic growth and development are significantly influenced by incubation temperature. Steroidogenesis in the gonad may be influenced by genetic factors and the rate of development. In *L. olivacea*, embryos develop more rapidly at the higher incubation temperature. Fast-developing gonads and Di produced more T than slow-developing gonads and Di at the male-promoting temperature. Interestingly, T concentration in serum was similar at both temperatures during TSP.

In the brain, on the other hand, our results in *L. olivacea* revealed significant differences in E2 contents, depending on the incubation temperature. Initially, we analyzed the steroid contents in whole brains (data not shown) and observed differences that led us to examine brain regions. Comparison of the Te and Di responses caused us to reject the possibility of a nonspecific response of neural tissues to temperature. In the Te, T and E2 contents did not show significant differences during TSP, but their concentrations increased slightly during the UDG and DG. On the other hand, in the Di, significant differences in T and E2 contents were observed in the three analyzed periods.

The gonad had T and E2 concentrations almost one order of magnitude higher than the brain. Thus, T and E2 in the brain could have been taken

up from the blood stream, thereby reflecting the steroids produced by the gonad. However, E2 concentrations detected in the serum of embryos incubated at female- or male-promoting temperatures did not show significant differences during TSP and UDG. In addition, the differences in E2 concentrations in the two brain regions make nonspecific tissue retention from the serum unlikely. Moreover, the biotransformation results demonstrated that, although the brain tissue has lower steroidogenic capacity than the gonad, it does possess steroidogenic enzymes. The presence of these enzymes in the brain makes it unlikely that the detected steroids represent a retention of hormones produced in another organ.

During the three periods, the Di showed a tendency to convert higher percentages of T into E2 at the female-promoting than at the male-promoting temperature. This correlated positively with the higher contents of E2 detected by RIA in Di. Furthermore, a positive correlation was also found between the higher E2 contents in Di than in Te and the higher tendency of Di to convert T into E2 than Te. Collectively, however, the data did not show a significant difference in the capacity for biotransformation between the tissues incubated at the male- or the female-promoting temperature. An explanation may be that, in the current experiments, only 1 h was allowed for the biotransformation of a low concentration of radioactive precursors by isolated organs in vitro, which compares to the days taken in vivo for accumulation of steroid hormones detected by RIA. It was assumed that the use of higher concentrations of radioactive precursors and/or longer incubation times would seriously disturb the physiological conditions.

In *L. olivacea*, we found that, as in other TSD species (Crews et al., '89; Dorizzi et al., '91; Wibbels et al., '92), E2 overrode the effect of the male-promoting temperature and feminized the gonad (Merchant-Larios et al., '97). It is probable that exogenous E2 alters the endogenous concentrations of this hormone in both gonad and Di. The female-promoting temperature favours greater production of E2 in Di than does the male-promoting temperature, and E2 production is similar in gonads at both temperatures, suggesting that the feminizing effect of exogenous E2 may occur at the brain level. Because experiments with aromatase inhibitors, aromatizable or non-aromatizable steroids, and other compounds (Crews et al., '89; Lance and Bogart, '92; Rhen and Lang, '94; Pieau et al., '94a, b; Wibbels and Crews, '92, '94) were administered to whole embryos, the results could also be interpreted by their effect on the brain.

The participation of the brain in sexual differentiation of the gonad can be indirect or direct. The indirect control would imply a mediation of the pituitary gland. Thus, factors released by the hypothalamus would stimulate gonadotropin secretion, which would in turn modulate gonadal differentiation and steroidogenesis. Unfortunately, to the best of our knowledge, no systematic studies are available on the ontogeny of the hypothalamus-pituitary-gonad (HPG) axis in reptiles. In the sea turtle *Caretta caretta,* Pearson et al. ('83) studied the ontogeny of the pituitary gland. Using immunohistochemistry, they found that cells containing ACTH, PRL, GH, LH, and TSH were present at the pouch stage of development of the pituitary gland. This stage occurred between days 20 and 30 in embryos incubated at 28–31°C. In *L. olivacea*, we have found that the TSP occurs between days 20 and 27 at both the male-promoting and the female-promoting temperature (Merchant-Larios et al., '97). Although species differences may exist, adenohypophyseal hormones can probably be produced in *L. olivacea* during TSP.

In *A. mississipiensis,* Deeming and Ferguson ('89) suggested that the neuroendocrine system could play an important role in sex determination by temperature. However, they did not provide experimental data to support their hypothesis. White and Thomas ('92b,c) found that in the turtle *T. scripta,* adrenal-kidney complexes (AKGs) respond sex-specifically to gonadotropins and other pituitary hormones. Although differences were evaluated in the steroidogenic capacity of AKGs in vitro, these results support the possibility that the brain could directly control the secretion of pituitary hormones in vivo, which would in turn regulate the steroidogenesis of AKGs.

Direct control of the brain over gonad differentiation implies the release of neurosecretory factors in situ. The presence of nerve terminals in the undifferentiated gonad of *L. olivacea* (Merchant-Larios et al., '89) allowed us to speculate on this possibility. Although in our previous report, the thermosensitive period had not been determined, recent observations confirm that nerve terminals can be found during this period (Merchant-Larios et al., unpubl. obs.). A paracrine effect of a neuroendocrine factor would be to act as morphogen in the undifferentiated gonad to induce differentiation into the ovaries or testicles. We hypothesized that in some neurons of the brain, a thermosensitive process may control the

expression of the aromatase gene. At a critical concentration of estrogen, a male-determining gene product is inhibited, and neural factors may directly or indirectly control the histological differentiation of the gonad. However, the origin of gonadal nerves, the kind of nerve terminals, and the nature of neurosecretions must be studied to support this hypothesis, and these questions are currently being explored in our laboratory. Our present results do not allow us to determine whether the diencephalon is the cause or the effect, but they conclusively demonstrate that this region of the brain senses temperature during sex determination *L. olivacea*.

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