

Aromatase Activity during Embryogenesis in the Brain and Adrenal–Kidney–Gonad of the Red-Eared Slider Turtle, a Species with Temperature-Dependent Sex Determination

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Gonadal sex in the red-eared slider turtle is determined by the incubation temperature that the embryo experiences during the mid-trimester of development. High temperatures result in female-biased sex ratios, and low temperatures produce male-biased sex ratios. The physiological equivalent of temperature appears to be a combination of the nature and abundance of steroidogenic enzymes and their products—including estradiol and its precursor, testosterone—and aromatase, the enzyme that converts testosterone to estradiol. Aromatase has been hypothesized to play a major role in the female developmental pathway in this species, and research in other species with temperature-dependent sex determination points to the brain as an organ that transduces the temperature signal into an aromatase response. In this study, we used a tritiated water assay to compare the pattern of estradiol biosynthesis at male- and female-producing temperatures in the brain and adrenal–kidney–gonad (AKG) through development. The pattern for both sexes in the AKG was one of increased activity after the temperature-sensitive period (TSP), but with no significant difference between sexes. In the brain, however, putative females exhibited a significantly higher level of aromatase activity than putative males at the beginning of the TSP, after which activity in both male and female brains decreased, dropping below detection in females

before hatch. These results point to the brain as a site of aromatase response to temperature in this species, and they suggest that the product of aromatase activity, estradiol, may induce alterations in the neuroendocrine axis controlling gonadal sex steroid hormone production.

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INTRODUCTION

In temperature-dependent sex determination (TSD) the temperature experienced by the egg during a relatively narrow window of development establishes the sex of the individual. Research on the red-eared slider turtle (*Trachemys scripta elegans*) led to the hypothesis that the physical stimulus of temperature is transduced into an endocrine signal that directs the sex-determination process: specifically, estrogens and their precursors, aromatizable androgens, stimulate the female-determining cascade and inhibit the male-determining cascade, whereas nonaromatizable androgens stimulate the male-determining cascade and inhibit the female-determining cascade (Crews *et al.*, 1994). Several findings from research on this species support this hypothesis for female sex determination. First, administration of exogenous estrogens and aro

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matizable androgens, such as androstenedione (AE) and testosterone (T), to eggs at an all male-producing incubation temperature leads to female development (see Crews, 1996 for review). The critical period for this effect overlaps with the temperature-sensitive period (TSP) (Wibbels *et al.*, 1991). Estrogenic ligands feminize via an estrogen-specific receptor (Wibbels and Crews, 1992) and the estrogen effect is dose dependent (Crews *et al.*, 1996). Additionally, administration of aromatase inhibitors blocks female development and allows male development at a female-producing temperature (Crews and Bergeron, 1994), and these same aromatase inhibitors also block aromatizable androgen-induced feminization when administered with T at a female-producing incubation temperature. Additionally, in the European pond turtle, another TSD species, treatment with aromatase inhibitors that results in male gonadal structure also results in concordant changes in aromatase activity in the gonad (Richard-Mercier *et al.*, 1995).

If incubation temperature results in temperature-specific steroid hormone profiles that in turn determine the sex of the embryo, then information about the activity of steroidogenic enzymes is critical to understanding TSD. Levels of aromatase activity should vary at some point during development based on the temperature of the incubating egg. There is abundant evidence that steroidogenic enzymes are present in the urogenital system of embryos of TSD species; however, much of this enzymatic activity is located in the adrenal and kidney of the red-eared slider turtle (Thomas *et al.*, 1992; White and Thomas, 1992a). Enzyme reaction product is not observed in the genital ridge at the beginning of the TSP at either incubation temperature, nor is it apparent in the differentiating gonads in embryos during the TSP; the only activity detected in the gonad is observed after the temperature-sensitive window (Thomas *et al.*, 1992; White and Thomas, 1992a). A similar pattern is also seen in the Olive Ridley sea turtle (*Lepidochelys olivacea*) (Salame-Mendez *et al.*, 1998), the American alligator (*Alligator mississippiensis*) (Smith *et al.*, 1994), and the saltwater crocodile (*Crocodylus porosus*) (Smith and Joss, 1994), but not in the pond turtle (*Emys orbicularis*) (Pieau *et al.*, 1999). This information suggests that in some TSD species, including the red-eared slider turtle, extragonadal tissues, and not the gonad itself, produce steroid hormones during embryogenesis.

Some studies point to the brain as a site of steroid hormone synthesis in response to temperature. Recent work by Salame-Mendez *et al.* (1998) measuring estradiol-17 β (E2) in the diencephalon/mesencephalon and telencephalon regions of the brain of the Olive Ridley sea turtle demonstrated differences between incubation temperatures during the TSP and thus between sexes. They found that the concentration of E2 was much higher in the diencephalon/mesencephalon of putative females than in those of putative males during the TSP. The researchers also report that E2 levels in the gonads of putative males and females showed no differences at any developmental period examined. Although these studies do not illustrate a mechanism by which neural differences are converted to gonadal differentiation, the different levels of steroid and enzyme activity before the onset of gonadal differentiation suggest that enzymes in the brain may play a role in establishing gonadal endocrine milieu specific to incubation temperature.

Because of the strong inference for the role of E2 and aromatase in sex determination in this TSD species, and in light of the data suggesting involvement of the brain, we analyzed the pattern of aromatase activity in the brains and adrenal-kidney-gonad (AKGs) of putative male and female red-eared slider turtles at stages from the beginning of the TSP to just before hatch.

MATERIALS AND METHODS

Egg Incubation/Tissue Harvesting

Freshly laid red-eared slider turtle (*T. scripta elegans*) eggs were obtained from a commercial supplier (Robert Kliebert, Hammond, LA) and brought back to the lab for processing. After being held at room temperature, eggs were candled to determine viability and then placed in groups of 35 in trays containing a 1:1 mix of vermiculite:water. Eggs from the same clutch were placed into different trays to avoid clutch effects. Trays were then placed in incubators set at either 26° (male-producing) or 31° (female-producing). In an effort to obtain as much data as possible, setting of eggs was staggered to obtain the same stage for several different assays. Eggs were held at 20° until they were

set. The embryonic stage at the time of setting for every egg was stage 13 or 14, well before the temperature-sensitive period (Wibbels *et al.*, 1991). Embryonic development was monitored by candling and dissecting representative eggs to verify developmental characteristics specific to particular stages (Yntema, 1968). All embryos were examined by two independent researchers to establish developmental stage. When embryos reached the appropriate stage (15, 16, 17, 18, 19, 20/21, or 23), the AKGs and brains were harvested for use in the tritiated water assay.

Tritiated Water Assay

The assay was adapted from Lephart and Simpson (1991) and serves as an indirect measure of aromatase activity. Briefly, aromatase, when converting testosterone to estradiol, cleaves a hydrogen in the process, which is then taken up by water molecules. This assay measures the amount of tritiated water that is a byproduct of aromatase conversion of testosterone to estradiol.

Embryos were quickly decapitated, and three tissue samples (either whole brain or AKG) were harvested and immediately placed into 300 μ l cold sucrose phosphate buffer (pH 7.4), so that in the final homogenate, 100 μ l would represent one tissue sample. For each stage and group, tissue was pooled to avoid individual effects; each homogenate contained tissue from three individuals. Tissue was homogenized with a hand-held homogenizer, and 100 μ l of homogenate, representing one average tissue sample, was transferred to its respective test tube. Two samples of each homogenate were assayed side by side. Prewarmed substrate media (glucose-6-dehydrogenase, Sigma; and phosphate buffer, pH 7.15) containing the substrate, testosterone (NEN Life Science Products, MA), at a final concentration of 200 nM and a cofactor mix (NAD, NADPH, glucose 6-phosphate, ATP; Sigma) were added in 150 μ l volumes per reaction tube. A blank containing substrate media and buffer, but no tissue, was also used. Red-eared slider female hatchling ovary served as the positive control.

Homogenate and substrate media were incubated in a shaking water bath at 37° for 1 h, after which the reaction was halted by addition of 5 \times volume of chloroform, which removed leftover steroid and denatured the protein. Deionized water was added to bring reaction volume to 1.5 ml. After centrifugation (1000

rpm; 10 min), the aqueous layer was removed and added to 1 ml cold charcoal:Dextran solution (5:0.5), which removed any remaining steroid. After a second centrifugation (3000 rpm; 30 min), 1 ml supernatant (one-third of sample) of each sample was counted in Ready-Safe (Beckman) scintillation fluid. The resulting dpms were corrected by subtracting the value of the blank, multiplying by 3 to obtain the total for the entire sample, dividing by the nanomolarity of the assay substrate (~200 nM) as determined by counting 150 μ l of the substrate/media mix, and taking an average of the two side-by-side samples.

Statistics

Data were analyzed with both ANOVAs and Student's *t* test using JMP statistical software for Macintosh (SAS Institute, Inc.). For statistical analysis, data were log-transformed to reduce heteroscedasticity.

RESULTS

The Brain

At stage 15, aromatase activity levels in the brain of both putative females (31°) and putative males (26°) were low and not significantly different. At stage 16, aromatase activity levels were significantly higher in brains of putative females (31°) than in the brains of putative males (26°) (ANOVA, *F* ratio = 11.54, *df* = 11, *P* = 0.006).

The pattern of expression in the brain also differed between the two temperatures (Fig. 1). In embryonic brains from 31° (female-producing), activity levels increased significantly from stage 15 to stage 16 (ANOVA, *F* ratio = 19.35, *df* = 14, *P* < 0.001) and decreased significantly from stage 17 to stage 18 (ANOVA, *F* ratio = 23.5263, *df* = 13, *P* < 0.001), the stage that marks the end of the TSP at this temperature. At stages 20/21, activity fell significantly below stage 15 levels (ANOVA, *F* ratio = 23.2163, *df* = 11, *P* < 0.001) and fell below detection at stage 23.

In embryonic brains from 26°, no significant changes in aromatase activity occurred from stage to stage from stages 15 to 23. Levels at stages 20/21 fell significantly below stage 15 levels (ANOVA, *F* ratio = 7.7026, *df* = 9, *P* = 0.02), but never fell below detection of the assay.

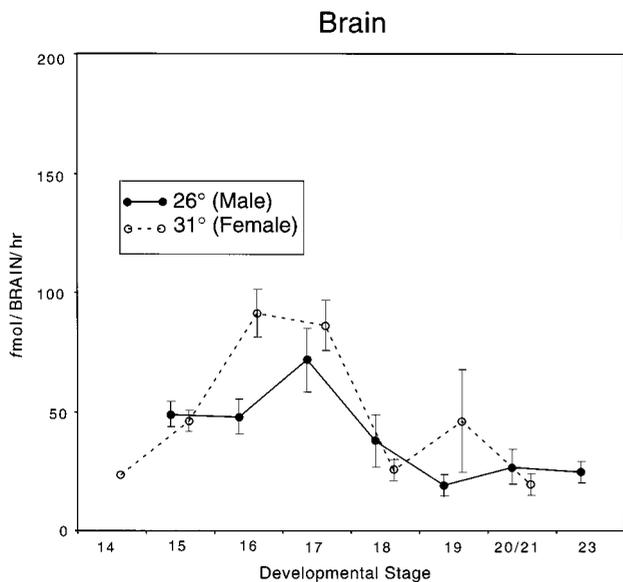


FIG. 1. Aromatase activity in fmol/brain/h in embryos incubating at female-producing (31°) and male-producing (26°) temperatures. Stages encompassing the beginning, middle, and end of the temperature-sensitive period are shown.

The Adrenal-Kidney-Gonad Complex

The pattern of aromatase activity in the AKG was similar for both temperatures (Fig. 2). At both temperatures, aromatase activity in the AKG was significantly higher after the TSP.

At a female-producing temperature (31°), the TSP ends at stage 18; AKG aromatase activity levels from stages 19–23 (post-TSP) were significantly higher than those of stages 15–18 (ANOVA, F ratio = 32.10, df = 42, P < 0.001) at 31°.

At a male-producing temperature (26°), the TSP ends at stage 19; AKG aromatase activity from stages 20–23 (post-TSP) was significantly higher than that of stages 15–19 (ANOVA, F ratio = 28.17, df = 42, P < 0.001) at 26°.

At no stage were there significant differences in activity levels in the two temperatures.

DISCUSSION

Information as to whether the endocrine environment of the embryonic gonad actually differs with incubation temperature in turtles with TSD is contradictory and appears to vary among species. Radioim-

unoassay of embryonic gonads from the European pond turtle (*Emys orbicularis*) reveals that during the TSP, gonads from embryos at a female-producing incubation temperature have higher estrogen content than gonads at a male-producing temperature (Dorizzi *et al.*, 1991). Further, aromatase activity is very low in undifferentiated gonads and remains low in embryos at male-producing temperature but increases exponentially in embryos at a female-producing temperature, an increase that begins several stages into the TSP (reviewed in Pieau *et al.*, 1999). In the red-eared slider, White and Thomas (1992a) report high levels of progesterone at the beginning and during the TSP, but could find no differences in hormone concentrations in the circulation of embryos at sex-specific temperatures. It is significant that the steroid content in the urogenital tissues (AKG) of embryos incubated at sex-specific temperatures also does not differ statistically (White and Thomas, 1992b). Additionally, steroidogenic activity in the slider appears to be confined primarily to the adrenal and kidney during TSP (Thomas *et al.*, 1992; White and Thomas,

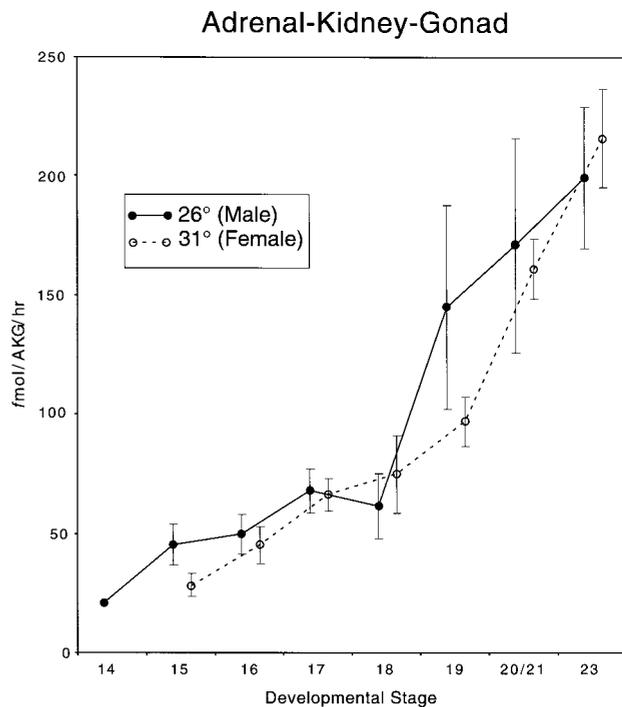


FIG. 2. Aromatase activity in fmol/AKG/h in embryos incubating at female-producing (31°) and male-producing (26°) temperatures. Stages encompassing the beginning, middle, and end of the temperature-sensitive period are shown.

1992b; E. Willingham and D. Crews, unpublished data) and not the gonad.

Yet the inference for the role of aromatase and estradiol in sex determination is powerful. In four different TSD species, administration of specific aromatase inhibitors to eggs incubating at a female-producing temperature results in male hatchlings (Crews and Bergeron, 1994; Dorizzi *et al.*, 1994; Rhen and Lang, 1994; Richard-Mercier *et al.*, 1995). In a number of TSD species, applying E2—the product of aromatase activity—to an egg incubating at a male-producing temperature causes females to result (Rhen and Lang, 1994; Wibbels *et al.*, 1992). Obviously, aromatase and/or E2 are involved in the female sex differentiation pathway.

Is there an extragonadal steroid source in TSD species? Research points to the brain as a possible sensor and transducer of temperature. In the diamondback terrapin (*Malaclemys terrapin*), aromatase transcripts are found in the brains of putative males and females prior to the TSP and are in greater abundance at the beginning of the TSP in females (Jeyasuria and Place, 1998). Later in embryogenesis, during TSP, transcript levels in the brains of putative males rise above those of females, while transcript levels in the gonads of putative females rise exponentially, results that recall those observed with aromatase activity levels in the current study. In the Olive Ridley sea turtle, E2 levels during the TSP are higher in the diencephalon/mesencephalon of putative females, but at all stages measured, no temperature differences in the gonads were identified (Salame-Mendez *et al.*, 1998). The results in the current study also point to the brain as a possible temperature sensor in the red-eared slider turtle. Studies examining the AKG for steroidogenic activity have failed to find differences during the TSP (White and Thomas, 1992), results supported by the current work, which found no temperature differences in aromatase activity in AKG.

However, the current data demonstrate differences in aromatase activity in the brain. The differences occur both in pattern of activity from stage to stage and in activity levels. Just as the TSP begins, embryonic brains from 31° (female-producing) exhibit a significantly higher level of activity (stage 16). The pattern for putative females (31°) shows a significant increase early in TSP followed by a significant decrease as the TSP ends at this temperature (stage 18). Putative males (26°) exhibit an increase later than that seen in females before decreasing at a rate comparable

to that observed in females. The strong inference from these data is that temperature affects aromatase activity in the brain at an important developmental period, and the higher temperature elicits a much different pattern of activity throughout development. Aromatase may be present in the brains of putative males and putative females, but a temperature-induced difference in pattern could be a determinant in whether an embryo commits to a male or female developmental pathway.

Although the AKG results do not point to any temperature differences in levels in these tissues, the patterns of expression do differ. Embryos from two temperatures used in this study experience a slightly different TSP. At 31° (female-producing), the TSP ends at stage 19; at 26° (male-producing), it ends at stage 21. The pattern of AKG aromatase activity for each temperature is similar in that levels after the TSP are significantly higher than those during the TSP. These data point to a role for aromatase in the AKG that may be temperature specific in terms of when the increase begins. Regardless of what is happening after the TSP, however, the current study demonstrates that the brain may be a site of aromatase activity at the beginning of the TSP in the red-eared slider turtle.

Why does the temperature-sensitive organ appear to differ among TSD species? Perhaps there really is no difference. The fact that, to our knowledge, the brain has not been examined in a number of species may be one reason that the mechanisms seem so disparate. In studies that demonstrate a temperature-related difference in aromatase activity in gonads or AKG complexes, these differences are always observed well into the TSP and during or after gonadal differentiation (Pieau *et al.*, 1999; White and Thomas, 1992a; Smith and Joss, 1994; Smith *et al.*, 1995). However, in all studies that implicate the brain—including the current study—differences are observed prior to or early in the TSP and well before gonadal differentiation (Salame-Mendez *et al.*, 1998; Jeyasuria and Place, 1998). It could be that the mechanisms are similar and signal transduction begins in the brain, with a subsequent transference of steroidogenic activity to the gonad, a pattern indicated in studies with the diamondback terrapin, in which transcript in brains of putative females is higher at the beginning of the TSP, but during TSP, transcript levels increase in the AKG (Jeyasuria and Place, 1998). The mechanism by which

this transfer occurs is not known, but may be neurally mediated (Crews, 1993).

The fact that, in this study, AKG levels of aromatase activity did not differ between temperatures could be explained in the same manner that Pieau *et al.* (1999) addressed similar results in the American alligator and saltwater crocodile (Smith *et al.*, 1995; Smith and Joss, 1994); that is, the gonads were not separated from the adrenal/mesonephros before assays were performed, and thus the activity of the adrenal and/or mesonephros could be masking subtle differences. White and Thomas (1992b) did find that the gonad of the red-eared slider turtle was quiescent during embryogenesis, a finding supported by current research (data not shown), but the activity of the mesonephros could still mask differences in activity of the adrenal or vice versa. Separating any of these tissues in the red-eared slider turtle embryo during TSP does not appear to be possible; so, these questions may have to be answered at the mRNA localization level. In addition to mRNA work, future studies involving shifting eggs from one temperature to another and treating eggs with E2 or aromatase inhibitor will help elucidate further the role of aromatase in the brain. But the findings of the current research point to the brain as an organ of interest in sex determination in this species.

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