Effects of Incubation Temperature and Estrogen Exposure on Aromatase Activity in the Brain and Gonads of Embryonic Alligators

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During embryogenesis, incubation temperature and the hormonal environment influence gonadal differentiation of some reptiles, including all crocodilians. Current evidence suggests that aromatase, the enzyme that converts androgens to estrogens, has a role in sexual differentiation of species that exhibit temperature-dependent sex determination (TSD). During the temperature-sensitive period (TSP) of sex determination, we compared aromatase activity in the brain and gonads of putative male and female alligator embryos to determine if aromatase activity in the embryonic brain could provide the hormonal environment necessary for ovarian development in a TSD species. In addition, we assessed the pattern of aromatase activity in the brain and gonads of embryos treated with estradiol-17β (E₂) and incubated at male-producing temperatures to compare enzyme activity in E₂ sex-reversed females to control males and females. This has particular significance regarding wildlife species living in areas contaminated with suspected environmental estrogens. Gonadal aromatase activity remained low during the early stages of the TSP in both sexes and increased late in the TSP only in females. Aromatase activity in the brain increased prior to gonadal differentiation in both sexes. These results suggest that aromatase activity in the brain is not directly responsible for mediating differentiation of the gonad. E₂ exposure at male-producing temperatures resulted in sex-reversed females that had intermediate gonad function and masculinized brain activity. This study indicates the need to examine multiple end points and to determine the persistence of developmental alterations in contaminant-exposed wildlife populations. 

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During embryonic development in reptiles, steroids influence sexual differentiation of the gonad and the brain. In many vertebrates, the presence or absence of sex-specific chromosomes ultimately determines sexual differentiation. In others, environmental influences such as temperature can be the pivotal factor in determining ovarian or testicular development. Temperature-dependent sex determination (TSD), in which egg incubation temperature determines the sex of the developing embryo, exists in many reptiles, including all crocodilians, most turtles, and some lizards (1). In the American alligator (Alligator mississippiensis), incubation temperatures near the low (30°C) and high (34.5°C) temperatures on sexual differentiation in the freshwater turtle Trachemys scripta (6) and alligators (7,8). This indicates that the undifferentiated gonad responds either directly to estrogen or indirectly by way of some estrogen-sensitive, extragonadal tissue.

The aromatase enzyme complex (aromatase cytochrome P450 and the flavoprotein nicotinamide adenine dinucleotide phosphate [NADPH]-cytochrome P450 reductase) is responsible for the conversion of androgens to estrogens. Aromatase activity has been detected in the gonad, brain, liver, and adipose tissue of many vertebrate species. The role of this steroidogenic enzyme is sex- and tissue-dependent, and varies according to the developmental stage of the organism. Most research on aromatase and TSD in reptiles has focused on gonadal aromatase activity. Treatment of eggs with aromatase inhibitors causes male development at female-producing temperatures in T. scripta (9) and prevents normal ovarian development in the alligator (10). However, gonadal aromatase, which exhibits increased mRNA expression and estrogen synthesis only near the end of the TSP in crocodilians (11–13) and turtles (14,15), does not appear to be the primary signal for ovarian development. The question remains, what is the normal signaling mechanism that causes ovarian development and how is this signal duplicated at male-producing temperatures in the presence of exogenous estrogens?

Recent research suggests that the brain plays a role in sexual differentiation in TSD species. Sexually dimorphic transcription of the aromatase gene has been detected in diamondback terrapin embryos (Malaclemys terrapin) during the early stages of sex determination, with a greater abundance of aromatase transcripts in the female brain (16). During the second half of the TSP, aromatase activity increases in the male brain to levels greater than those in the female brain (16). Willingham et al. (14) measured aromatase activity in the brains of male and female T. scripta embryos and found activity levels in female brains that were higher than those in males at the beginning of the TSP. Aromatase activity of both sexes decreased following the end of the TSP and dropped below detection levels in females prior to hatching (14). In contrast to the sexually dimorphic brain aromatase expression reported in turtles, no significant differences were found in brain mRNA of alligator embryos incubated at male- and female-producing temperatures (13). Although substantial evidence implicating the brain in directing gonadal differentiation is lacking, temperature appears to influence sexual differentiation of the brain during embryonic development in some TSD species.

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Like several TSD species of turtles in which low doses of estrogenic compounds cause the development of female offspring at male-producing temperatures, the alligator has become a model for screening environmentally relevant concentrations include \( \alpha, \beta \)-DDE, \( \alpha, \beta \)-DDD (17), \( \alpha, \beta \)-DDT (18), and \( \alpha, \beta \)-DDE (19). Although the mechanism by which these compounds influence sexual differentiation is poorly understood, all show some affinity for the alligator estrogen receptor (aER) (20). The herbicide atrazine shows a weak affinity for the aER (20) and causes testicular aromatase activity uncharacteristic of males or females but does not cause sex reversal (8).

Although the feminizing action of estrogenic compounds has been well documented in terms of gonadal morphology in alligators, little is known about the functional consequences of chemically induced sex reversal. Field studies of several contaminated lakes in Florida have shown a number of functional abnormalities in female alligators, including elevated ovarian synthesis of testosterone, elevated hepatic degradation of testosterone, and reduced ovarian synthesis of estradiol-17\( \beta \). The mechanism by which these compounds act is poorly understood, all show some affinity for the alligator estrogen receptor (aER) (20). Because nothing is known concerning the incubation conditions of the animals obtained for these studies, it is unknown if any were sex reversed as a result of embryonic contaminant exposure. The possibility exists that the differences observed in the exposed populations are due in part to altered endocrine function in sex-reversed females.

Given the uncertainty of the mechanisms and consequences of chemically induced sex reversal, we conducted an initial study to examine the timing and levels of aromatase activity in the brain and gonads of putative female, male, and sex-reversed female alligator embryos. Our purpose was to determine if sexual dimorphism in whole-brain aromatase activity could provide a means of directing gonadal differentiation and to compare aromatase activity in the brain and gonads of E\(_2\)-sex-reversed females to that in the brain and gonads of untreated males and females.

**Materials and Methods**

**Animals and Tissue Collection**

Six clutches of alligator eggs were collected from Lake Woodruff National Wildlife Refuge, Volusia County, Florida, within the first 2 weeks postoviposition. Eggs were transported to the University of Florida (Gainesville, Florida, USA) and incubated in damp sphagnum moss at an intermediate temperature of 32°C until reaching embryonic stage 19. Fifteen eggs from each clutch were systematically assigned to three treatment groups and three dissection stages within each treatment group to avoid clutch effects within the experiment. Treatment groups consisted of control females incubated at 30°C, control males incubated at 33.5°C, and sex-reversed females incubated at 33.5°C and were treated topically with 90 µg E\(_2\) dissolved in 50 µL 95% ethanol at stage 19. Previous studies show alligator eggs incubated at male-producing temperatures treated with similar doses of E\(_2\) result in 100% female hatchlings (2.7,8). Additional eggs (three to four per clutch) were incubated at each temperature to verify the appropriate stages for dissection of each clutch.

Ten embryos from each treatment group were selected for dissection at stages 20 (early TSP), 22 (middle TSP), and 24 (late TSP). Upon reaching the appropriate stage, embryos were decapitated immediately upon removal from the egg. Brains and paired gonad–adrenal–mesonephros complexes (GAMs) were removed, flash frozen in liquid nitrogen, and stored at −70°C until assayed. Entire GAMs were used because of the difficulty in separating the three tissues; published research shows that the majority of aromatase activity takes place in the gonad portion of the complex (12).

**Brain Aromatase Activity**

The tritiated water assay used to measure aromatase activity was a modification of methods described by Lephart and Simpson (22) and Willingham et al. (14). All buffers and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified. Whole brains or paired GAMs were homogenized over ice in 100 µL homogenate buffer (RPMI-1640 culture medium supplemented with 25 mM Heps and 1 mM dithiothreitol) in microcentrifuge tubes using a handheld pellet pestle (Kontes, Vineland, NJ, USA). Tissue homogenates were transferred to glass culture tubes along with 400 µL substrate buffer. Substrate buffer consisted of homogenate buffer supplemented with 1 mM NADPH, 10 mM \( \alpha, \beta \)-d-glucose 6-dehydrogenase, 1 U/mL glucose 6-dehydrogenase, and 0.8 µM \([\alpha-\beta] H\) androstenedione (DuPont NEN Research Products, Boston, MA, USA). Culture tubes were covered with parafilm and incubated on a shaker at 32°C.

Following 9 hr of incubation for brains and 6 hr for GAMs, 1.5 mL chloroform was added to halt the reaction. The volume of the aqueous phase was brought up to 900 µL with the addition of 400 µL deionized water. Culture tubes were then pulse vortexed and centrifuged at 2,000 × g for 15 min. A 600-µL aliquot of the aqueous phase was transferred to a new tube, and 600 µL of 5% charcoal/0.5% dextran was added before the tube was vortexed and centrifuged at 2,000 × g for 15 min. Five milliliters of scintillation fluid (Scintillation BD, Fisher Scientific, Pittsburgh, PA, USA) was added to 300 µL supernatant, and the tube was counted on a Beckman scintillation counter (Beckman Instruments, Schaumburg, IL, USA).

Aromatase activity is proportional to the amount of tritiated water produced by the cleavage of hydrogen from androstenedione at the 1β position. Activity was calculated by multiplying the sample decays per minute (dpm) by 3, subtracting the background (blank tube), and dividing by the dpm of substrate originally added. This percentage was then multiplied by the mass of substrate added and expressed as fmol/tissue/hr. Sensitivity of the assay was defined as twice the mean dpm of blank tubes, which corresponded to approximately 8 fmol/tube/hr.

**Statistics**

Statistical analyses were performed with StatView for Windows (2.3). Single-classification analysis of variance (ANOVA) was used to test for differences across stages within a treatment group and among treatment groups within a stage. A two-way ANOVA was not performed as part of the analysis because a comparison of all possible combinations of stage and treatment was not consistent with the purpose of this study. Fisher’s protected least significant difference was used to make pairwise comparisons, with the level of statistical significance set at \( p \leq 0.05 \).

**Results**

**GAM Aromatase Activity**

No difference in GAM aromatase activity (Figure 1) was detectable among treatment groups at stage 20 (\( p = 0.302 \)), and no differences were detectable between stages 20 and 22 within any treatment group. However, enzyme activity at stage 22 in control females was lower than that in control males (\( p = 0.012 \)) and sex-reversed females (\( p = 0.014 \)). Stage 24 was marked by a dramatic increase in enzyme activity in control females (\( p < 0.0001 \)), whereas control males exhibited a slight decrease in aromatase activity (\( p = 0.022 \)) from stage 22. A moderate increase in aromatase activity was detected in \( E_2 \)-treated females (\( p = 0.0009 \)) that was higher than that in control males (\( p = 0.0001 \)) and lower than that in control females (\( p < 0.0001 \)) at stage 24.

**Brain Aromatase Activity**

Brain aromatase activity (Figure 2) increased from stage 20 to 22 in all treatment groups,
and no differences among treatment groups were detectable at these two stages ($p = 0.359$ and $0.806$, respectively). From stage 22 to stage 24, aromatase activity increased in control males ($p = 0.011$) and females ($p = 0.001$); no difference was detected between these two groups at stage 24 ($p = 0.084$). No change in aromatase activity occurred in sex-reversed females from stage 22 to 24 ($p = 0.631$); activity in stage 24 sex-reversed females was lower than that in control females ($p = 0.013$) and was not different from that in control males ($p = 0.363$).

**Discussion**

Similar to previous studies (11,12,14,15), aromatase activity in the GAM did not increase until the end of the TSP. It is likely that gonadal aromatase activity is associated with ovarian development, as it increased significantly between stages 22 and 24 in both control and sex-reversed females. The proliferation of cortically located germ cells and regression of medullary sex cords occur during these stages in alligators incubated at female-producing temperatures (3). However, temperature-shift experiments by Lang and Andrews (2) show sex determination to occur between stages 20 and 22 when shifting from 30 to 33°C. Aromatase activity alone does not appear to initiate ovarian differentiation, as evidenced by the low activity in both males and females during the early stages of the TSP.

The data presented in this study indicate that the GAM of sex-reversed females is neither malelike nor femalelike with regard to aromatase activity at stage 24. This is especially interesting because E$_2$ exposure at male-producing temperatures results in ovarian differentiation, as opposed to an intersexed gonad (7,9). That aromatase activity in the sex-reversed females was significantly lower than that in control females and higher than that in control males suggests embryonic exposure to E$_2$ and incubation temperature affect steroidogenic enzyme levels and/or activity. Apart from directing ovarian differentiation of the gonad, exogenous estrogen could disrupt a number of feedback mechanisms along the hypothalamic–pituitary–gonadal axis, such as gonadotropin release, causing suppression of aromatase synthesis relative to control females (4). Incubation temperature, regardless of sex, influences plasma steroid concentrations. In the red-eared slider turtle, plasma E$_2$ in females from intermediate incubation temperatures was significantly lower than that in juveniles from the all-female–producing temperature, and no different than that in males from the intermediate temperature (24). This effect could be mediated by the presence of anti-Müllerian hormone (AMH), which decreases aromatase synthesis in fetal ovaries of several mammal species (25). Western et al. (26) detected expression of AMH in alligator embryos incubated at male-producing temperatures at stage 22 but not at female-producing temperatures at any stage. AMH expression was limited to the medullary cells of the developing testes (26), indicating a need to examine ovarian differentiation of sex-reversed females on a morphological level (e.g., in situ hybridization for AMH mRNA) relative to that in untreated embryos, as well as to measure multiple hormones.

Results of recent studies on aromatase in the brain of TSD species have differed according to species and end points measured. In the diamondback terrapin, transcripts of the aromatase gene were in greater abundance in females during the first half of the TSP, then higher in males during the second half (16). When aromatase enzyme activity was measured in the brain of red-eared slider turtle embryos, females exhibited an increase early in the TSP, whereas males showed no significant increase throughout the same period (14). In the alligator, transcripts of the aromatase gene did not differ between sexes and showed no significant increase for any stage of development (13). In contrast, our data indicate an increase in enzymatic activity throughout the TSP in both sexes, with slightly higher activity in putative females at stage 24, indicating that gene expression does not necessarily reflect enzyme activity. Furthermore, E$_2$-induced sex reversal resulted in brain activity similar to that in control males, suggesting that sex-reversed females do not function as normal females on all levels.

Because an increase in aromatase activity occurred early in the TSP but did not differ between sexes, it is difficult to determine the role of brain aromatase activity with regard to sex determination. If the increase in brain aromatase activity is sufficient to increase circulating E$_2$ concentrations, temperature-dependent expression of the estrogen receptor (ER) could be the key to gonadal differentiation. That is, a slight increase in circulating E$_2$ resulting from aromatase activity in the brain early in the TSP, coinciding with an increase in ER expression in the gonad, could lead to ovarian differentiation. Bergeron et al. (27) measured ER transcripts in the gonads of red-eared slider turtle embryos and found higher concentrations in the gonads of embryos incubated at female-producing temperatures at the beginning of the TSP. However, it is not known if the estrogen produced locally in the brain is capable of crossing the blood–brain barrier to an extent great enough to affect circulating steroid concentrations. Furthermore, translation of ER transcripts to functional receptor proteins should be confirmed before strong inferences are made regarding the interplay of aromatase activity and ER expression in sex determination.

In contrast to the results from the gonads, levels of aromatase activity in the brain of sex-reversed females are not different from those of control males, indicating E$_2$ exposure did not override the effects of incubation temperature on enzyme activity in the brain. Studies on eutherian mammals have shown that the presence of α-fetoprotein, which binds to circulating E$_2$, prevents maternal E$_2$ from crossing the blood–brain barrier of embryos developing in utero (28). Although α-fetoprotein has not been reported in any reptile, cytosolic-binding proteins have been described in the alligator that show an affinity for E$_2$ and, to a lesser extent, synthetic steroids and contaminants (29). Conley et al. (30) reported high concentrations of steroids (E$_2$, testosterone, androstenedione) in alligator egg yolks that decline significantly during the TSP. The presence of steroid-binding proteins in developing embryos could function as a means to protect the embryo from high

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**Figure 1.** GAM complex aromatase activity (fmol/GAM/hr) in female (30°C), male (33.5°C), and sex-reversed female (33.5°C + E$_2$) alligator embryos during the early (stage 20), middle (stage 22), and late (stage 24) stages of the TSP.

**Figure 2.** Brain aromatase activity (fmol/brain/hr) in female (30°C), male (33.5°C), and sex-reversed female (33.5°C + E$_2$) alligator embryos during the early (stage 20), middle (stage 22), and late (stage 24) stages of the TSP.
concentrations of maternal steroids deposited in the yolk during vitellogenesis and prevent feminization of the brain following embryonic exposure to exogenous E2.

Estradiol-exposure studies serve as valuable models but cannot always predict the effects of estrogenic contaminants because the pathways through which these compounds work vary widely. Although many of the environmental estrogenic compounds that cause sex reversal are capable of binding to the aER (20), many differ from natural estrogens in hepatic degradation rates, binding affinity for plasma proteins, and binding affinity for other nuclear and membrane-bound receptors. As the result of E2 exposure differed between the brain and gonad in this study, special consideration should be given to which end points are monitored in organisms exposed to estrogenic compounds. Estrogens and aromatizable androgens have been shown to override the effect of incubation temperature on sex determination (7–9), but few studies have looked beyond gross morphology of the gonad. Egg-dosing studies in which embryos are exposed to environmentally relevant concentrations of contaminants have produced alterations similar to those reported in exposed wildlife populations. For example, alligator embryos exposed to ecologically relevant concentrations of various pesticide metabolites exhibit sex reversal but also have altered gonadal steroidogenesis and gonadal enzyme activity (18,19).

In the present study, E2 exposure at male-producing temperatures resulted in intersexed gonadal and malaleike brain aromatase activity in female embryos. Although our study did not examine specific brain regions, this initial study demonstrates that the gonad and brain respond to differing degrees after exogenous estrogen treatment. This is extraordinary, considering that the response in any given region of the brain would be tempered by the fact the entire brain was homogenized. Future studies should examine aromatase activity in distinct regions of the brain associated with sexual differentiation, such as the preoptic area and the hypothalamus. Further research is warranted to determine if alterations in enzyme activity occur following contamination-induced sex reversal, and whether they persist in light of the endocrine alterations reported in field studies of exposed alligator populations. This data clearly demonstrate that environmental contaminants could alter the differentiation of the gonad morphologically while having only partial influence on the differentiation of gonadal physiology. Moreover, gonadal differentiation could be affected differently from the response seen in the brain. Such differences could be associated with the timing of exposure or exposure dosage as modified by physiological phenomena such as the transfer of chemicals across the blood–brain barrier. Because of the pervasive influence of the hypothalamic–pituitary–gonadal axis in numerous endocrine activities including reproduction, growth, and metabolism, understanding the effects of environmental estrogens and antiestrogens is essential if we are to determine the impact these compounds have on development and reproduction of many wildlife species. Only a thorough assessment at the tissue, cellular, and molecular levels can determine the full impact of a chemically altered embryonic environment.

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