

# Alligator Aromatase cDNA Sequence and Its Expression in Embryos at Male and Female Incubation Temperatures

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**ABSTRACT** In all species of crocodylians, sex is determined not by genetic mechanisms, but by the temperature at which the egg is incubated. In the American alligator (*Alligator mississippiensis*) the thermosensitive period (TSP) for sex determination is a 7- to 10-day window within stages 21–24 of development, around the middle third of the incubation period. Treating embryos with estrogen during the TSP produces female offspring, even at male incubation temperatures. Conversely, blocking embryonic estrogen synthesis at female-inducing temperature prevents development of the female phenotype. Therefore, it has been suggested that estrogen plays a role in determination of sex in the alligator. Estrogen is produced from an androgen substrate by cytochrome P450 aromatase (CYP19). If estrogen plays a critical role in sex determination, there should be differences in aromatase expression between embryos at male- and female-producing temperatures during the TSP. Therefore, to address this question, we cloned and characterized the alligator CYP19 cDNA. Based on the sequence information, a quantitative kinetic reverse transcriptase–polymerase chain reaction (TaqMan) assay was designed to measure expression of the alligator aromatase gene in RNA extracted from the gonadal and brain regions of alligator embryos incubated at male- or female-producing temperatures from prior to the TSP through hatching. Aromatase expression was detected in the brain region from the earliest stage tested (stage 20) through hatching. The hypothalamus had significantly higher expression than the forebrain or hindbrain in both male and female embryos. Expression was not significantly different in the gonadal region between embryos at male and female temperatures until after the TSP, when there was a dramatic increase in expression at female temperature. These data indicate that aromatase expression and, thus, estrogen production, are not the initial trigger for sex determination but play an essential role in ovarian differentiation in the alligator. *J. Exp. Zool.* 290:439–448, 2001. © 2001 Wiley-Liss, Inc.

In mammals, birds, and some reptiles, sex is determined by genetic mechanisms. Heteromorphic sex chromosomes are present in female birds, some snakes and lizards, and male mammals. In many species of reptiles, including most turtles (Ewert and Nelson, '91) and all crocodylians studied thus far (Lang and Andrews, '94), there are no discernible sex chromosomes. Sex is determined not by the presence or absence of specific genes, but by the temperature at which the eggs are incubated—temperature-dependent sex determination (TSD). In the American alligator (*Alligator mississippiensis*), 100% male offspring are produced by incubation at a constant temperature of

33°C and 100% female offspring are produced by incubation at temperatures from 29°C to 31.5°C (Ferguson and Joanen, '82; Lang and Andrews, '94). Increasing percentages of female offspring are produced above 34°C, with 95% females being produced at 34.5°C. Survivorship decreases markedly at temperatures above 34°C, until no embryos survive when incubated at 36°C (Lang and Andrews,

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'94). The critical period during which temperature determines sex is 7–10 days between stages 21 and 24 of embryogenesis, referred to as the thermosensitive period (TSP) (Lang and Andrews, '94). The molecular mechanism by which temperature determines sex in crocodylians has not yet been elucidated.

Histologically discernible differentiation of the gonad begins during the TSP (Smith and Joss, '93). Treatment with estrogen of alligator embryos incubated at a temperature that normally produces 100% males results in 100% female hatchlings (Lance and Bogart, '91). Treating eggs with an aromatase inhibitor to block estrogen synthesis inhibits ovarian development in embryos incubated at female-producing temperature, though the gonad is not totally masculinized (Lance and Bogart, '92). In turtles (Dorizzi et al., '94) and birds (Elbrecht and Smith, '92), however, complete masculinization of the gonad has been achieved by treating eggs with an aromatase inhibitor. These data indicate that estrogen is necessary for female gonadal development in these species and that, in the absence of estrogen, a testis develops.

The enzyme that catalyzes estrogen production from an androgen substrate is cytochrome P450 aromatase (CYP19) (Simpson et al., '94). It was suggested that, if estrogen plays a role in temperature-dependent sex determination, there should be differences in aromatase activity at male- and female-producing temperatures during the TSP (Bogart, '87; Desvages and Pieau, '92). Smith et al. ('95) measured aromatase activity in the adrenal-kidney-gonad complex (AKG; also known as the gonad-adrenal-mesonephros, or GAM) of developing alligator embryos by incubating the minced tissue with [ $1\beta$ - $^3\text{H}$ ] androstenedione and measuring tritiated water production. Using this method, they found no significant differences in aromatase activity in the AKGs of male versus female embryos during the TSP. However, after the TSP, there was a dramatic increase in aromatase activity in the female AKG, but activity at male temperature remained low (Smith et al., '95). These results would suggest that aromatase and, thus, estrogen, does not play a role in the initial determination of sex in the alligator, but only comes into play later during differentiation of the ovary. In contrast, differences in aromatase activity in male and female gonad regions have been detected toward the end of the TSP in the European pond turtle, another species with TSD (Desvages and Pieau, '92).

It is possible that, early in the TSP, very small

amounts of estrogen are produced in the differentiating gonad but act only in a local or paracrine way. If this is the case, differences in aromatase activity might be well below the threshold of detection of the tritiated water assay. Very slight differences in aromatase expression, on the other hand, might be detectable by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR), a very sensitive technique for determining the amount of mRNA present for a given gene (Lockey et al., '98).

Aromatase transcripts have been detected in significant amounts in the brain, as well as in the gonad, of most vertebrate species (Simpson et al., '94). Jeyasuria and Place ('98) suggested that aromatase expression in turtle brain might play a role in TSD.

The aim of this study was to use RT-PCR to measure aromatase expression in the AKG and brain of alligator embryos incubated at male- or female-inducing temperatures, thereby directly answering the question of whether estrogen plays an important role in the determination of sex in this species.

## MATERIALS AND METHODS

Alligator eggs ( $n = 230$ ) were collected from the Rockefeller Wildlife Refuge in Louisiana within 1–2 days of being deposited in the nest. Eggs were transported to the San Diego Zoo, where they were held at constant male ( $33^\circ\text{C}$ ) or female ( $29$ – $31^\circ\text{C}$ ) incubation temperatures (see Lance and Bogart, '92, for details). Embryos were sacrificed at regular intervals from stage 10 of development through hatching, and the AKG was dissected out, immediately frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until processed. For some early-stage embryos, the whole embryo ( $n = 16$ ) or the torso region ( $n = 51$ ) was collected, because of the difficulty of fine dissection at early stages. For later-stage embryos, the entire AKG was collected ( $n = 94$ ), or the gonad was roughly separated from the adrenal-mesonephros ( $n = 23$ ). Alligator embryos were sacrificed by decapitation in accordance with a protocol approved by the animal use committee of the Zoological Society of San Diego. Brains were also collected from embryos beginning at stage 20 through hatching ( $n = 107$ ), and 87 of these were dissected into forebrain, hindbrain, and hypothalamic regions. Heart, kidney, lung, stomach, liver, fat body, and muscle tissues were also collected from a hatchling. Tissues were homogenized using an Ultra-Turrax homogenizer and TriPure isolation reagent (Boehringer-Mannheim,

Indianapolis, IN) according to manufacturer's protocol. First-strand cDNA synthesis was performed on 1 µg of total RNA using MMLV-RT (Life Technologies, Gaithersburg, MD) and a poly dT primer with a GC-clamp (5'-T<sub>(16)</sub>(ACG)N-3') to reverse transcribe polyA+ mRNA. All primers used in this study were synthesized by the BioAnalytical Services Laboratory at the Center of Marine Biotechnology in Maryland. Primer annealing was carried out at 70°C for 10 min, before reverse transcriptase was added. First-strand synthesis conditions were 42°C for 50 min, followed by 15 min at 70°C.

Ovarian tissue was collected from a female alligator killed by a hunter in Louisiana and immediately frozen in liquid nitrogen. The tissue was stored at -80°C until processed. A cDNA library from mRNA from the tissue was prepared in lambda ZAP II (Stratagene, San Diego, CA) as described (Blumberg et al., '91). A degenerate oligonucleotide (YTT CAT CAT NAC CAT NGC DAT) corresponding to the reverse complement of a highly conserved region of the published CYP19 sequences (I A M V M M K) was used to screen the cDNA library using the tetramethylammonium chloride method (Blumberg et al., '91). The identity of positive clones was verified by sequence analysis using the degenerate oligonucleotide as a primer.

As the cDNA library produced only truncated clones of the gene (see Results), RACE (Rapid Amplification of cDNA Ends) was used to obtain a full-length coding sequence for the alligator aromatase gene. Ovarian tissue harvested from a wild-caught alligator during the breeding season with high circulating levels of estradiol (390 pg/ml) (Lance, '89) was ground to a powder under liquid nitrogen using a mortar and pestle. mRNA was isolated with the Poly (A) Pure kit (Ambion), and first-strand synthesis was performed using 500 ng of the mRNA and the 5' RACE System for Rapid Amplification of cDNA Ends (Life Technologies). An alligator aromatase gene-specific primer (GSP 1 arom: 5'-GAATAGTGACCAAACCTG-3') was used for primer extension. Following reverse transcription, the cDNA was purified and an oligo-dC tail was added to its 5' end as per manufacturer protocol.

PCR amplifications were performed using 2.5 U of AmpliTaq DNA polymerase in 1X buffer II, 200 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 5 µl of the dC-tailed RACE reaction in a final volume of 50 µl. Primers included 0.4 µM Abridged Anchor primer (Life Technologies) and 0.4 µM alligator

aromatase gene-specific primer 2 (GSP 2 arom: 5'-TGACACTTGCCCTGTAATGCTTC-3'). A hot start method using AmpliWax PCR gems (Perkin Elmer) was used to improve the specificity of the reaction. The thermocycling profile was denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 10 min.

Ten percent of the amplified product was electrophoresed on a 1° agarose gel containing ethidium bromide. The aromatase RACE PCR product was excised from the gel, purified with the UltraClean Gel Purification Kit (Mo Bio Laboratories), and cloned into the TA vector pCR 2.1 (Invitrogen). Plasmid DNA was isolated from positive colonies using the SNAP MiniPrep kit (Invitrogen), and 1 mg was subjected to restriction enzyme digestion in a reaction containing 10 U *Eco* RI and 1X buffer H (Promega). Digested plasmid DNA was run on a 1° agarose gel, then transferred overnight to Hybond N+ membrane (Amersham) using standard methods. The blot was fixed in a UV Stratalinker (Stratagene) and prehybridized in ECL gold buffer (Amersham) for 1 hr at 42°C. An 800-bp partial aromatase cDNA clone isolated from the alligator ovarian library was labeled using the ECL direct system (Amersham) and hybridized overnight with the blot as per manufacturer's recommendations. The blot was washed, reacted with ECL detection solution (Amersham), and then exposed to Hyperfilm ECL (Amersham).

Plasmids containing the longest RACE PCR products were sequenced using the fluorescent dye termination method on an Applied Biosystems 373 DNA sequencer. Both strands of DNA were completely sequenced using a primer walking technique. The forward primers were: T7 (Invitrogen), 5'ER 1 (5'-GTGCCCATACTCCTCCTCAT-3'), and #187 (5'-ATGCGACCTTTCTTCACCAA-3'). The reverse primers were: M13 Reverse (Invitrogen), #117 (5'-TGGCAATAAACTTCCCTACA-3'), and AR Rev (5'-GTTTGGCATGTCATCGCTCTGTA-3'). Sequences were aligned using the AssembLIGN program (Genetics Computer Group).

Primers for Quantitative PCR were designed from the full-length alligator aromatase coding sequence to amplify a region of 139 bp. A partial sequence was also obtained for the alligator β-actin gene for the purpose of normalization, and primers were designed based on this sequence to amplify a region of 105 bp. TaqMan probes (PE Biosystems) were also designed for β-actin (5'-VIC, 3'-TAMRA) and aromatase (5'-FAM, 3'-TAMRA)

to hybridize across an inferred intron/exon boundary to reduce the likelihood of amplifying genomic DNA (Fig. 1). PCR was carried out in the ABI prism 7700 according to manufacturer's protocol, with the exception that one fourth of the suggested reaction volume (25  $\mu$ l) was used. PCR conditions were 2 min at 50°C, 95°C for 10 min and 50 cycles at 95°C for 15 sec, and 60°C for 1 min. All samples were run in duplicate, and the mean  $C_T$  (threshold cycle) for actin was subtracted from the aromatase mean  $C_T$  for normalization. Results were converted into fold change over basal expression, which was defined as the average expression in all of the gonad samples during the TSP, since almost no expression was seen prior to the TSP and there was no difference in expression between males and females until after the TSP ( $P = 0.37$ , during TSP between male and female temperatures). A sample of pooled cDNA was included as a calibrator in each TaqMan assay to ensure constancy of results. The interassay variability was low, since results varied no more than 1.1 cycles of PCR between assays. A sample containing no probe was also included in each run to control for contamination. No contamination was noted in any of the runs.

Statistical analysis was carried out using StatView SE+ Graphics statistical software. All statistics are factorial ANOVA at the 95% confidence level.  $P$ -values given are from the  $F$ -test.

## RESULTS

To identify the coding sequence of the alligator aromatase gene, a cDNA library was constructed from ovarian mRNA. The library was screened with the degenerate oligonucleotide (YTT CAT CAT NAC CAT NGC DAT) corresponding to the reverse complement of a highly conserved region

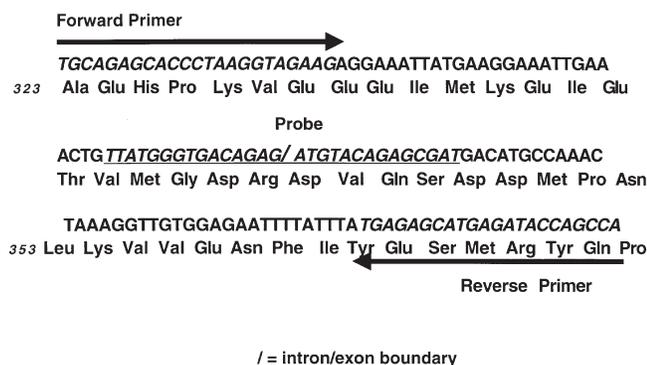


Fig. 1. Region of the aromatase gene amplified by TaqMan PCR. The TaqMan Probe was designed to hybridize across an intron/exon boundary.

of the published CYP19 sequences (I A M V M M K). Sequence evaluation of the positive clones revealed that they were all truncated, with the longest insert containing approximately 800 bp. Therefore, to obtain the remainder of the 5' gene sequence, RACE PCR was performed. Following the PCR amplification utilizing the RACE reactions as templates, the products were purified and cloned. To identify plasmids containing the full 5' RACE PCR product, restriction digested plasmid DNA was subjected to Southern blot analysis using the 800-bp alligator aromatase cDNA clone as a probe. Plasmids containing appropriately hybridizing restriction fragments were selected for double-stranded cycle sequencing using a primer walking strategy. The full-length alligator aromatase cDNA sequence (Accession # AY029233) is 1,542 nucleotides, coding for a protein of 504 amino acids (Fig. 2).

To gain further insight into the evolutionary relationships between P450 aromatase proteins, a phylogenetic tree was constructed using the Phylogeny Inference Program, Version 3.572c (Fig. 3) (Felsenstein, '93). The alligator aromatase falls within the bird/reptile cluster and shares the highest homology with birds.

The results from the quantitative RT-PCR amplification of embryonic alligator mRNA indicate that aromatase expression in the AKG was affected by both stage of development and temperature at which the embryo was incubated (Table 1, Fig. 4). There was very little aromatase expression in the AKG at either male or female temperature prior to the TSP (average was 13.98-fold higher than basal for female temperature and 1.75-fold higher than basal for male temperature), and the difference between male and female temperature expression was not significant at this stage ( $P = 0.38$ ). Expression in the AKG did not increase significantly at female or male temperature during the TSP ( $P = 0.80$  and  $0.09$  for female and male, respectively, before vs. during the TSP). After the TSP there was an increase in aromatase expression at both female and male temperatures, which was significant for both ( $P = 0.02$ ), but much larger at female-producing temperature ( $P = 0.04$  for female vs. male expression after the TSP).

Average expression in female AKGs after the TSP was  $223.43 \pm 100.39$  times higher than that of basal expression, while male expression was only  $11.77 \pm 5.6$  times that of basal. Of total AKG samples collected after the TSP, 18 out of 20 female samples and only 7 out of 32 male samples had expression at least two times that of basal

CTCAGCAACAAAATCTGCAAGGGTCTGGAAGGTACTGATTACAGTTAAAGGTGCTTCAAATTTGAAT  
 CCGTCTGCCCTCTTTTCCCAATTGCTTCCACAGAAAGCAAAGGAAATTCCTCTGAAAAG

ATGATACTGGAACCTTGAATCCAATGCATTACAACATCACCAATGTGGTGCCAGAAGCC  
 1 M I L E T L N P M H Y N I T N V V P E A  
 ATGCCAGTGGCCACCGTGCCCATCTCCTCCTCATGGGCTTTCTTTTCCATAATGGAAT  
 21 M P A A T V P I L L L M G F L F L I W N  
 TATGAAGAAACATCATCAATCCCAGGGCCTTGTATTGTATGGGAATCGGTGCCCTCATT  
 41 Y E E T S S I P G P C Y C M G I G A L I  
 TCACATGGGAGATTTCTTTGGATGGGAGTAGGCAATGCCTGCAACTATTACAATAAGATA  
 61 S H G R F L W A  
 TATGGAGAATTTGTGAGAGTTTGGATCAGTGGAGAGGAAACACTTATTATTAGCAAATCC  
 81 E F V R V W I S G E E T L I I S K  
 TCAAGTGTGTCTATGTAATGAAACATGGCCACTATGTCTCTAGATTTGGGAGCAAGCTT  
 101 B Y V S R F G S K L  
 GGGTTACAGTGTATTGGCATGTATGAAAATGGTATTATATTTAATAATAATCCAACACTT  
 121 B' E N G I I F N N N  
 TGGAAA GAAATGCGACCTTTCTTACCACAA GCTTTGTCTGGTCCTGGCTTGTACGTATG  
 141 C P F F T K A L S G  
 ATA TCAATTTGTGTGAATCAACAACAGATCATCTGGACAAA TTGGAAGAGGTGACCACT  
 161 D L E E V  
 GAACTGGGAAACATCAATGTGTTGAACCTTATGAGGCGGATCATGTTAGATACTTCTAAC  
 181 E N  
 AAACTCTTTCTCAGGATCCCTTTGGATGAAAATGCTATTGTGCTTAAAATTGAGAACTAC  
 201 K L F L R I P L F  
 TTTGATGCCTGGCAAGCACTTTTACTGAAA CCTGACATCTTTTCAAGATTCTTGGCTG  
 221 L L K P D I F F K I S W  
 TACAAGAAA TATGAGGAGTCAAGATTTGAAA GGAGCAATGGATTATTAATACAG  
 241 G  
 CAGAAA CGTCAGAAGCTTCCACTGTTGAAAAA CTGGATGAGCACGTGGACTTTGCAACA  
 261 K L S T V E K L D E H V D  
 CAACTGATATTTGCACAGAAACCGTGGAGATCTGACAGCTGAAAACGTTAACCAAGTGTG  
 281 H Q N R G D L  
 CTGGAGATGATGATCGCTGCCCTGACACACTATCAGTGACTCTCTTCTCATGCTTGT  
 301 I  
 CTGATTGCAGAGCACCCCTAAGGTAGAAGAGGAAATTATGAAGGAAATTGAAACTGTTATG  
 321 J' J  
 GGTGACAGAGATGTACAGAGCGATGACATGCCAAACCTAAAGGTTGTGGAGAATTTTATT  
 341 G D R D K  
 TATGAGAGCATGATACACAGCCAGTTGTGGACTTATCATGCGTAAGGCTCTACAGGAT  
 361 P V V D L I M R K A L Q D  
 GATGTGATCGATGGCTATCCTGTGAAA AAGGGGCAAAACATTATTCTGAACATTGGACGA  
 381 D V I D G Y P V K K G T N I I L N K'  
 ATGCATAAGCTTGAATCTTTCCAAAGCCTGATGAGITTTCTCTTGAATAATTTGAGAAG  
 401 K L E F F P K P D E F S L E N F E K  
 AGTGTCTCTCTCGTATTTT CAGCCATTTGGATTTGGCCCTCGGGCGTGTGTAGGGAAG  
 421 S V P S R Y F Q P F G F G P R A C V G K  
 TTTATTGCCATGGTAATGATGAAA GCAATA TTGGTGA CACTTCTGAGACGGTGCAGAGTA  
 441 L R C R V  
 CACACACAGAAA GGAAGAGGCTAAACAACATCCAGAAA AACAATGACTTATCCATGCAT  
 461 H T Q K G R G L N N I Q K N N D L S M H  
 CCAAATGAAAGGCAGCCCTTACTTGAGATGGTTTTACACCAAGAAGCATTACAGGCAAG  
 481 P N E R Q P L L E M V F T P R S I T G K  
 TGTGAGGGTACTAG  
 501 C Q G D \*  
 TAACAGATGACAAGTGGTGTGAGTTCTAGGGCTCTCATTGCCCTAGTCCAACTGATAAAAGCCCAACCTT  
 CCTAAAGGAAGTCATGATTTCAATTAACCTAAACATATTTCTGCCCTTAATATATGAGCACTTTAGGTGAT  
 CACCTACTTTGCCCTGAAGAGCTAAACAGGTTTGGTCACTATTCAAAGCTGACAAACAAGTTCAAGTAT  
 ATACTCAAGTGCAGTTGGTGAAGTGTCAATGTGAAAATGTAATTTTAAAGAAACACACCGTCTTGA  
 AGAGATTAGGCTCTGCTGTTTCACTGCAAGCTCAAGTCAATGACTGCAGACTTGAAGCAAAAGGGT  
 TAGGGTTTTCTCTCTATAGTGAAGGGATGCTTTTAGCAGAAGCTCAAAGAGACATCATCAGTTGTGTA  
 CTGTATAAGAGCTTAATAACTGTCTCAGAACCAATTTAATAAAATATTAACCAATGTTCAAAAAAAA  
 AAAAAAAA

Fig. 2. Alligator aromatase cDNA and deduced amino acid sequence. The message strand nucleotide sequence of the alligator aromatase gene with its corresponding amino acid translation is diagrammed. The amino acids are numbered with the methionine of the translation initiation codon desig-

nated as 1. The stop codon is indicated by \*. The shadowed regions represent the helices in the predicted structure of aromatase. The GenBank accession number of this sequence is AY029233.

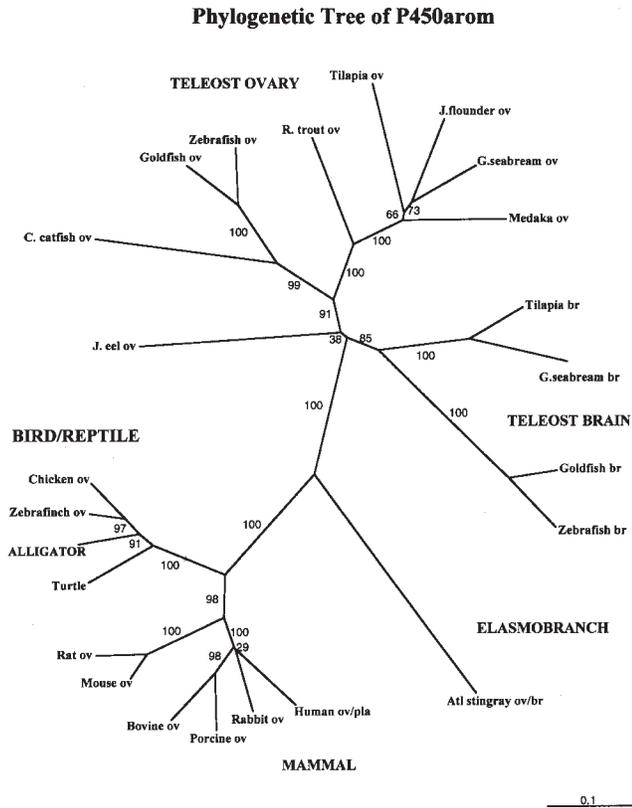


Fig. 3. Evolutionary relationships of the known P450arom. The phylogenetic tree was calculated using the neighbor-joining method based on the Kimura protein distance method using the PHYLIP program (Felsenstein, '93). Comparisons were made to the amino acid sequences of human placenta/ovary, bovine placenta/ovary, rabbit ovary, porcine ovary, mouse ovary/placenta, rat ovary/placenta, chicken ovary, zebrafinch ovary, turtle (Jeyasuria and Place, '98), Atlantic stingray, Japanese eel ovary, and Gilthead seabream brain and ovary (personal communication from Dr. Shigeo Ijiri).

expression. The fact that average aromatase expression at female-inducing temperature was higher than at male-inducing temperature prior to and during the TSP is due to only one individual in each time period with expression over 300-fold higher than basal expression. All other samples had expression less than 20-fold higher than basal. Most female temperature embryos at

these stages had expression at similar levels to the male temperature embryos. There was low but detectable aromatase expression in the torso region of some of the earliest embryos collected (stage 7), which had just been collected from the nest and were not yet held at a male or female temperature.

Data obtained by TaqMan assay of brain regions indicate that aromatase expression was different in the three tissues, but it was not linked to temperature of incubation. There were no significant differences in expression in any of the brain tissues between male and female incubation temperatures for any stage of development. Expression in the hypothalamus appeared to increase through the period of incubation (Fig. 4), though this increase was not statistically significant ( $P = 0.11$ ). Across stages, expression in the forebrain was higher than in the hindbrain ( $P = 0.02$ ), and expression in the hypothalamus was higher than for any of the other tissues (average fold change =  $190.74 \pm 31.87$  for hypothalamus,  $79.95 \pm 15.20$  for forebrain,  $38.06 \pm 6.81$  for hindbrain,  $P = 0.0001$ ) (Fig. 5). There was low but detectable aromatase expression in the earliest brain samples collected (stage 7). Brain expression at both male and female temperature after the TSP was at levels similar to female AKG aromatase expression (Fig. 6). Average brain expression was  $103.97 \pm 12.81$  higher than basal expression, while expression in the gonad was an average of  $49.85 \pm 20.95$  higher than basal. Levels of aromatase expression in the liver, muscle, heart, lung, and stomach did not exceed basal expression.

## DISCUSSION

The deduced amino acid sequence of alligator aromatase shows close homology to that of the birds and the turtle. Paleontological and protein sequence data strongly support the close affinities of birds and crocodylians (Wang and Conlon, '93), but the close relationship of the chelonians and crocodylians is more controversial (Lee, '97).

TABLE 1. Fold change in aromatase expression in the alligator AKG during and after the TSP<sup>1</sup>

	During TSP			After TSP		
	n	Mean $\pm$ SEM	Range	n	Mean $\pm$ SEM	Range
Female	28	19.76 $\pm$ 17.35	0–487	20	223.43 $\pm$ 100.39	0–1,863
Male	28	0.41 $\pm$ 0.21	0–4.94	21	11.77 $\pm$ 5.6	0–78.68

<sup>1</sup>Differences between expression in female temperature AKGs during and after the TSP were statistically significant ( $P = 0.02$ ), as were differences between male and female temperature AKGs after the TSP ( $P = 0.04$ ). There were no significant differences in aromatase expression during the TSP ( $P = 0.12$ ).

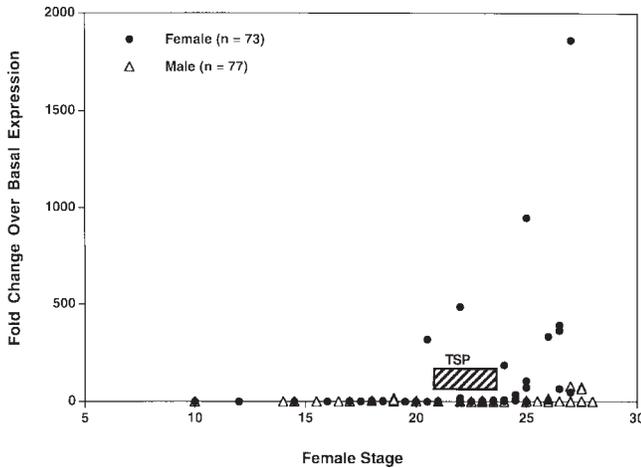


Fig. 4. Aromatase expression in the AKG (or torso region, for early embryos) of alligator embryos incubated at male- and female-producing temperatures, as determined by TaqMan RT-PCR. There was low but measurable expression in this region for some of the earliest embryos collected, but, with the exception of one AKG at female temperature with a very high fold change in expression (319 times higher than basal) and one male embryo with a fold change 17.19 times higher than basal, no embryo had a fold change more than five times over basal expression, and most (33/45) had expression less than one fold higher than basal expression. There was no significant increase in aromatase expression in the AKG until after the TSP, when aromatase expression increased significantly at both female and male temperatures ( $P = 0.02$ ) but much more dramatically in female temperature embryos. It was only after the TSP that there was a significant difference between aromatase expression in male and female temperature embryos ( $P = 0.04$  for female vs. male expression after the TSP).

There is, however, substantial molecular evidence that the Chelonia and the Crocodylia are not, as previously thought, distantly related separate lineages, but are in fact derived from a common ancestor within the last 200 million years (Rieppel and deBraga, '96; Zardoya and Meyer, '98; Hedges and Poling, '99; Mannen and Li, '99). The close sequence similarity of the turtle and alligator aromatase supports this interpretation.

The data presented here demonstrate that expression of CYP19 is temperature and stage dependent during embryogenesis in the American alligator. Increased CYP19 expression corresponds with the onset of female gonadal differentiation, following the thermosensitive period (TSP). These results agree with those obtained by Smith et al. ('95) for aromatase activity in the developing gonadal region of the alligator, suggesting that increased expression is, in fact, leading to increased aromatase activity. Substantial amounts of testosterone and androstenedione have been found in

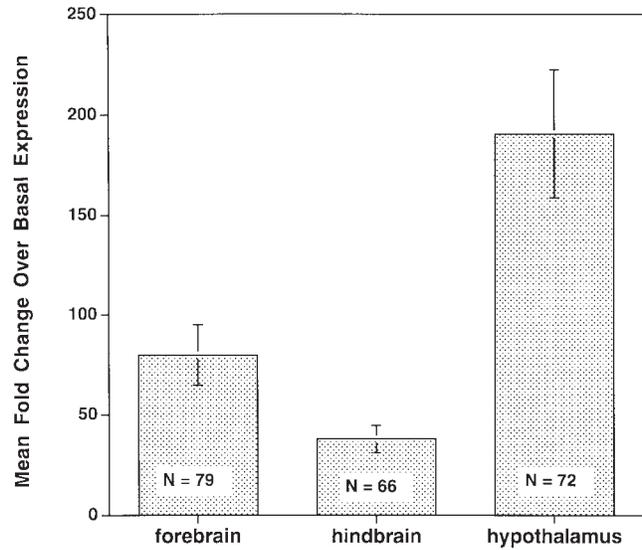


Fig. 5. Average aromatase expression in forebrain, hindbrain, and hypothalamus during and after the TSP. There were no significant differences in expression in any of the brain regions corresponding to stage of incubation ( $P = 0.11$ ).

the yolk of alligator eggs; therefore, sufficient substrate is potentially available for aromatase to act on to form estradiol (Conley et al., '97). Our observation that there is no significant difference in aromatase expression between male and female temperatures until after the TSP indicates that aromatase is a downstream component of the female sexual differentiation process. Therefore,

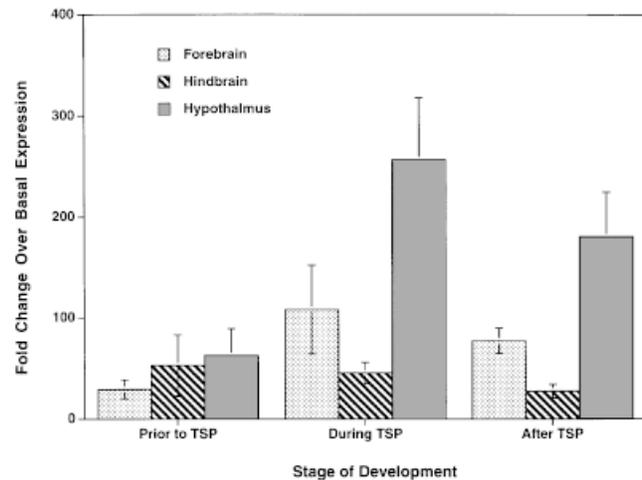


Fig. 6. Average aromatase expression in forebrain, hindbrain, and hypothalamus regions during the entire incubation period. Expression in the hypothalamus was significantly higher than in all other tissues ( $P = 0.0001$ ). There were no significant differences in expression between any of the other tissues ( $P = 0.19$ ).

there must be a mechanism which lies upstream of aromatase transcription that is directly influenced by incubation temperature to lead to the stimulation of aromatase production in the female or its corresponding suppression in the male developing gonad.

The fact that there were a few individuals at female-inducing temperature which began to express high levels of aromatase before and during the TSP could be due to the genetic differences in response to temperature. There is evidence for genetic differences in sensitivity to temperature among alligator eggs. For instance, different clutches show different hatchling sex ratios when incubated at the pivotal temperature: a temperature that should produce a 50:50 sex ratio (Lang and Andrews, '94).

It was proposed by Smith et al. ('95) that aromatase production occurs constitutively during embryogenesis and that it is suppressed at male-producing temperature. Since female development occurs at both high and low incubation temperatures, it is conceivable that there is some factor that is turned on or off at intermediate (male-producing) temperature during the TSP and that suppresses post-TSP aromatase production to allow development of the male phenotype. A protein which may play a role in regulation of aromatase production is the orphan nuclear receptor, steroidogenic factor 1 (*SF1*). Western et al. (2000) found that while *SF1* expression in *A. mississippiensis* embryos, as determined by RT-PCR, remained constant through stages 20–27 at female incubation temperatures, levels in male temperature embryos were downregulated during stages 23–25 (Western et al., 2000). This corresponds to the period during which aromatase transcript levels are increasing. Furthermore, *SF1* is known to play a role in regulating aromatase in mammals (Fitzpatrick and Richards, '93; Lynch et al., '93). Given that an *SF1* binding region has been found in the aromatase gene of mammals, birds, and fish (Matsumine et al., '91; Takayama et al., '95; Tanaka et al., '95; Parker and Schimmer, '97), it is likely that this motif will also be found when the complete sequence is obtained for the alligator aromatase gene. If this is the case, downregulation of *SF1* at male-promoting temperatures during the TSP might inhibit aromatase transcription that would otherwise increase constitutively after the TSP. In the absence of aromatase expression, testis differentiation would occur.

Another possible regulator of aromatase is anti-

Müllerian hormone (*AMH*), which has been shown to inhibit aromatase biosynthesis in rat (Clemente et al., '92), sheep, and rabbit ovarian tissue in vitro (Vigier et al., '89). In mammals, *AMH* is expressed at much higher levels in male than in female gonadal tissue and has been shown to cause regression of the Müllerian duct. *AMH* is expressed during embryogenesis in the alligator in the developing testis but not in the ovary (Western et al., '99). Since *AMH* expression in the male developing gonad slightly precedes the increased aromatase expression seen in the female, it is possible that *AMH* is working to antagonize aromatase production that would otherwise also be increased in the male.

It has been suggested that aromatase expression in the brain affects gonadal differentiation in TSD reptiles (Jeyasuria and Place, '98). When alligator gonads were cultured independently of the embryo and incubated at male or female temperature, they showed no sign of differentiation (Lance and Bogart, '94). This result is consistent with an extragonadal site of sex determination and regulation. When gonads of the olive ridley sea turtle were cultured independently of the embryo and incubated at male- or female-producing temperature, differentiation was consistent with the temperature at which the whole embryo was incubated before explantation, rather than differentiating according to the temperature at which just the gonad alone was cultured (Merchant-Larios and Villalpando, '90). The notion of the brain being the site at which TSD occurs, however, remains controversial. Normal gonadal differentiation has been seen after decapitation of early embryos of two non-TSD lizard species (see Raynaud and Pieau, '85), indicating that the brain is not necessary for gonadogenesis to occur in these reptiles. The results presented here confirm that aromatase is present throughout the TSP in the brains of embryos incubated at both male and female temperatures, but there are no significant differences in aromatase expression between male- and female-producing temperatures in any of the regions of the brain. This is different than the case of the diamond-back terrapin, in which sex-specific differences in aromatase expression in the brain that correspond to the TSP have been found (Jeyasuria and Place, '98). The data presented here for expression of aromatase in the brain of *A. mississippiensis* are not consistent with temperature-dependent control of aromatase expression in the developing alligator brain.

Sex determination is a complex process involv-

ing the interaction of many gene products, some of which are only just beginning to be understood. Estrogen production by the enzyme aromatase has been shown to play a crucial role in ovarian differentiation in birds and in reptiles with temperature-dependent sex determination. However, expression of aromatase does not appear to be the initial trigger for sex determination in these species. It is more likely that some upstream mechanism is directly affected by temperature to initiate a gene cascade, leading to upregulation of aromatase production in female embryos following sex determination. The study of upstream activators of aromatase may lead to useful insights in understanding temperature-dependent sex determination.

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