Aromatase enzyme activity during gonadal sex differentiation in alligator embryos

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Abstract. Sexual differentiation of the gonads in Alligator mississippiensis and many other oviparous reptiles is controlled by egg incubation temperature. Estrogens are thought to play a role in this process, and it has been hypothesized that estrogen production is thermosensitive in species with temperature-dependent sex determination (TSD). Using the tritiated water assay, we measured the activity of the critical estrogen-synthesising enzyme, P450 aromatase, in the gonad-adrenal-mesonephric kidney complex (GAM) of alligator embryos incubated at male- and female-producing temperatures. Aromatase activity increased in the GAM of developing embryos incubated at 30°C (100% female-producing) and 34.5°C (predominantly female-producing), while it remained very low throughout development in embryos incubated at the intermediate temperature of 33°C (100% male-producing). However, it is unclear whether enhanced aromatase activity represents the initial signal for ovary differentiation or whether it lies downstream in the female developmental pathway. For embryos incubated at 30°C (female-producing), there was no detectable increase in aromatase activity until developmental stage 24, which is after the temperature-sensitive period for sex determination. This suggests that aromatase may be a downstream component of the ovary-determining cascade. In female alligator hatchlings, most of the aromatase activity was localised in the ovary, activity being low in the adrenal-mesonephros. Aromatase assays carried out at 30°C and at 33°C indicated that, at viable incubation temperatures, aromatase activity is not thermosensitive. This suggests that temperature directly or indirectly influences enzyme synthesis. Radioimmunoassay of estradiol synthesised by the GAM during the aromatase assay confirmed increased enzyme activity during female development but not during male development. Increased aromatase activity and estrogen synthesis during female development were correlated with the timing of ovary differentiation, particularly proliferation of the gonadal cortex. These findings implicate aromatase in temperature-dependent gonadal sex differentiation in alligator embryos, higher enzyme activity being associated with ovary development.

Introduction

Despite some major advances in recent years, the mechanisms underlying gonadal sex differentiation in vertebrates are still largely unknown. A role for steroid hormones in gonadogenesis was first suggested over 50 years ago [32, 57, 59]. Many studies have subsequently examined the effects of androgens and estrogens on the development of the gonads [2, 4]. The so-called hormonal theory of gonadal differentiation, however, has not been completely supported. While the treatment of larval fishes and amphibians with androgens or estrogens can induce testis or ovary differentiation, respectively, [2, 58, 61], it is unclear whether sex steroids play a natural role in gonadal morphogenesis in these vertebrates. Among amniotes, a role for androgens in gonadal differentiation has not been convincingly shown, whereas there is considerable evidence implicating estrogens in gonadogenesis, at least in birds and reptiles. In bird embryos, exogenous estrogens can transiently feminise the gonads of genetic males, while the antiestrogen, tamoxifen, can masculinise the gonads of females (see [40]). The gonads are more steroidogenically active in female chicken embryos than in males [21], and gonadal estrogen levels are higher in developing ovaries than in testes [40, 60].

In many species of reptiles, sex is determined environmentally by egg incubation temperature ([5, 9, 18, 48], reviewed in [16, 24, 38]). In the American alligator, Alligator mississippiensis, high and low incubation temperatures produce mostly or exclusively females, while intermediate temperatures produce males [30]. Estrogens applied to eggs incubated at male-producing temperatures can induce ovary differentiation and female development in alligators and other reptiles with temperature-dependent sex determination (TSD) [6, 8, 14, 22, 35,
36]; reviewed in [7] and [53]. Antiestrogens such as tamoxifen can masculinise the gonads (induce seminiferous cord differentiation) at female-producing temperatures in some species [14] (but tamoxifen may act as an estrogen agonist in alligator [27]). Estrogens can also feminise the embryonic gonads or induce complete ovarian differentiation among those reptiles in which sex is determined genotypically [6, 38].

In species with TSD, the sex-determining mechanism is sensitive to temperature during a critical period of embryogenesis. This temperature-sensitive period (TSP) has been identified for each species by shifting eggs from the male- to female-producing temperature, and vice versa, throughout incubation and examining the sex ratios of resulting hatchlings. The TSPs appear to coincide with the onset of gonadal sex differentiation [36, 43, 51]. Furthermore, sensitivity of gonadal differentiation to exogenous estrogens occurs prior to and/or during the TSPs [6, 8, 23, 52]. Estrogen is therefore implicated in the process of gonadal sex differentiation in species with TSD.

Recent studies point to a key role for the estrogen-synthesizing enzyme P450 aromatase in the ovary-determining pathway of birds and reptiles. Treatment of genetically female chicken embryos with a specific aromatase inhibitor can induce sex reversal [15, 50]. In reptiles with TSD, aromatase inhibitors applied to eggs incubated at the female-producing temperature can disrupt ovary differentiation [28] or induce male development at temperatures that otherwise yield both sexes [39, 55]. It has been hypothesised that aromatase enzyme synthesis is thermosensitive in species with TSD, higher levels being produced at female-determining temperatures, leading to enhanced estrogen production and ovary differentiation [4, 10], reviewed in [36]). The present study examines this hypothesis by investigating aromatase enzyme activity in the urogenital tissues of Alligator mississippiensis embryos during development at male- and female-producing temperatures. Changes in aromatase activity are related to the timing of gonadal sex differentiation.

**Methods**

**Incubation of eggs and staging of embryos.** Seven clutches comprising over 200 fertile eggs of Alligator mississippiensis were obtained soon after oviposition from wild nests located on the Rockefeller Wildlife Refuge, southwestern Louisiana. The eggs were transported by road to the University of North Dakota, Grand Forks, where they were divided into three groups and incubated in foam box incubators [31] at either 30° C, 33° C or 34.5 °C. Incubation at 30° C and 33° C results in 100% female development and 100% male development, respectively [18, 26, 27, 30]. Initial studies indicate that incubation at 34.5°C yields over 90% females ("high temperature females"), although embryonic mortality increases at this temperature [30]. Embryos incubated at the three temperatures were examined before (developmental stage 19), during (stage 21) and after (stages 23, 24, 25 and 28) the temperature-sensitive period (TSP) for sex determination [30]. All embryos were weighed and then staged according to Ferguson [17], prior to excision of the paired gonad-adrenal-mesonephros complexes (GAMs). Aromatase assays were carried out on the entire GAM because of the difficulty in separating the three components until late in embryogenesis.

**Aromatase assay.** The tritiated water assay [1, 47] was used to measure aromatase activity in whole GAMs incubated in vitro, as described previously for crocodile embryos [42]. Paired GAMs dissected from individual embryos were incubated at least in triplicate in 400 μl HEPES-buffered RPMI-1640 culture medium, 7.2, supplemented with 3 mg/ml glutamine (Sigma, St. Louis, USA) and 0.8 μlM tritiated steroid substrate ([1β-3H]androstenedione, 24.1 Ci/mmol; New England Nuclear). For some stage-21 embryos, in which enzyme levels proved to be very low, pools of six pairs of GAMs per replicate were also assayed. Tissues were incubated for 6 h in a shaking water bath set at the corresponding egg incubation temperature. (For some developmental stages, the direct effect of temperature on aromatase activity was examined by carrying out assays at different temperatures, as described in Results). At the end of the incubation period, unreacted tritiated steroid was removed by chloroform extraction and dextran-charcoal adsorption, as described previously [42]. Aromatase activity was calculated from the rate of tritium released from the 1β-position during the aromatisation of [1β-3H] androstenedione into estrogens. The tritium, released as 3H2O, is recovered in the aqueous phase. Results are expressed as fmol/GAM/h or fmol/mg protein/h. The sensitivity of the assay was defined as twice the mean cpm of blank tubes, ~300 cpm, corresponding to an aromatase activity of 1.5 fmol/h. When assays were carried out in the presence of 100 μM 4-OH-androstenedione, a competitive steroidal inhibitor of aromatase, release of 3H was virtually abolished, demonstrating the specificity of the assay for the aromatase enzyme.

Following the aromatase assay, one GAM from each pair was processed for histology, as described below. The contralateral GAM was snap-frozen in liquid nitrogen and stored at −70° C prior to assaying for total soluble protein. The bicinchoninic acid (BCA) method (Pierce [45]) was used to measure protein content following homogenisation of GAMs in phosphate-buffered saline (PBS). Bovine serum albumin (BSA) was used as the standard.

**Radioimmunoassay of estradiol produced during aromatase assays.** To confirm the production of estrogens during the aromatase assay, the chloroform and dextran-charcoal used to remove unreacted tritiated androstenedione were retained, extracted and assayed for any synthesised estradiol for stage-23, -25 and -28 embryos. (For technical reasons, samples from earlier stages were not included). Firstly, the chloroform phases from triplicate tubes were pooled and set aside. Secondly, the charcoal pellets from these triplicates were extensively vortexed in the presence of fresh chloroform and centrifuged at 2000 g. The chloroform layers were removed, combined and then added to the pooled charcoal set aside (above). The total volume of chloroform, containing pooled steroids from triplicate tubes, was then dried down under nitrogen and the steroids reconstituted in 1 ml 100% ethanol. Samples were stored at 4°C prior to radioimmunoassay for estradiol-17β. Samples were firstly dried under nitrogen and reconstituted in 550 μl assay buffer (0.1% gelatin in PBS). The samples were allowed to equilibrate overnight at 4°C, and duplicate 200-μl aliquots were then assayed for estradiol using modifications of a highly specific, commercially available 125I-estradiol-17β RIA kit (Diagnostic Products Corp, Los Angeles, CA, USA) previously described [46]. Separation of bound versus free steroid was accomplished by the addition of 500 μl dextran-coated charcoal in assay buffer, incubation at room temperature for 15 min, followed by centrifugation at 3000 g, 4°C, for 15 min as described by Fivizzani et al. [19]. Supernatant aliquots of 500 μl were counted in a Beckman 5500 gamma counter. The sensitivity of this assay is 0.0625 pg/assay tube, and the interassay and intraassay coefficients of variation are 11.98% and 11.11%, respectively. Results are expressed as pg of estradiol produced/GAM during the 6-h aromatase assay incubation period.

**Gonadal histology.** Unilateral GAMs used for measurement of aromatase activity were retained, fixed in glutaraldehyde, post-fixed in osmium tetroxide and processed for high-resolution light microscopy, as described previously [41]. Semi-thin sections (1 μm)
were stained with 1% toluidine blue. In addition, some ultra-thin sections of gonads from high-temperature female hatchlings (34.5°C) were stained with uranyl acetate and lead citrate and examined at the ultrastructural level.

Results

Aromatase activity

Aromatase enzyme activity was detectable in the GAM throughout embryogenesis at both male- and female-producing temperatures. Detectable enzyme activity in the GAM of female embryos incubated at either 30°C or 34.5°C increased from stage 24 to hatching, whereas aromatase activity remained very low throughout development in the GAM of male embryos incubated at 33°C (Fig. 1). The increase in detectable aromatase activity in presumptive female GAMs occurred after the temperature-sensitive period (TSP) for female sex determination at 30°C, as defined by Lang and Andrews [30] and shown in Fig. 1. In younger embryos sampled earlier than stage 24, aromatase activity in the GAM was very low at 30°C, 33°C and 34.5°C (approximately 8 fmol/GAM/h) and was not significantly different between the three temperatures (ANOVA, P>0.05 for stages 19, 21, and 23). Even when six rather than two pairs of GAMs were pooled for assay of aromatase, the overall activity was still very low and there were no significant temperature-dependent differences in the levels of detectable aromatase activity until after the TSP (i.e. after stage 23).

Table 1 shows the mean soluble protein content of the GAM and aromatase activity/mg protein in the GAM over development at 30°C, 33°C and 34.5°C. For each stage examined, the protein content of the GAM was similar at the three temperatures. Expressed per mg protein, aromatase activity actually decreased over stages 19 to 23 at each temperature. However, it increased after stage 23 in presumptive females, but not in presumptive males, consistent with the pattern of aromatase activity/whole GAM (Fig. 1). Since aromatase activity in the whole GAM was low but stable over stages 19 to 23, decreasing activity/mg GAM protein was probably due to the rapid growth of the mesonephric kidneys relative to the gonads during the first half of incubation.

At stage 25, aromatase activity in the GAM of “high-temperature females” (34.5°C) was lower than that in the GAM of “low-temperature females” (30°C), but by stage 28 (hatching) levels were similar in both groups (Fig. 1). (Histological analysis showed that the stage-

![Fig. 1. Aromatase activity in the gonad-adrenal-mesonephros complex (GAM) of alligator embryos during development at 30°C (0), 33°C (●) and 34.5°C (○), females; □ males). The temperature-sensitive period for female sex determination at 30°C is shown (TSP-30°C). Values are means±SEM](image1)

![Fig. 2. Aromatase activity in the gonad (●) and adrenal-mesonephros (□) of female and male Alligator mississippiensis hatchlings incubated at 34.5°C](image2)

### Table 1. Mean soluble protein content (mg) and aromatase activity (fmol/mg protein/h) in the gonad-adrenal-mesonephros (GAM) or Alligator mississippiensis embryos over stages of development at 30°C, 33°C and 34.5°C

<table>
<thead>
<tr>
<th>Stage</th>
<th>Mean total protein (mg)</th>
<th>Mean aromatase activity (fmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>0.56</td>
<td>13.21</td>
</tr>
<tr>
<td>21</td>
<td>0.79</td>
<td>8.10</td>
</tr>
<tr>
<td>23</td>
<td>0.74</td>
<td>7.45</td>
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<tr>
<td>25</td>
<td>1.31</td>
<td>209.23</td>
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<tr>
<td>28</td>
<td>1.19</td>
<td>354.03</td>
</tr>
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</table>
25/34.5°C embryos used for aromatase assay were indeed all females). Two of six embryos examined at stage 28/34.5°C were males; aromatase activity in the GAMs of these males was low and comparable to that of males incubated at 33°C (Fig. 1). When the gonad could be separated from the adrenal-mesonephros in some female hatchlings incubated at 34.5°C, most aromatase activity was found to be in the gonad (ovary), while the very low levels in males were similar in both the gonad (testis) and the adrenal-mesonephros (Fig. 2). Higher levels of aromatase activity appeared to be present in the adrenal-mesonephros of female hatchlings than in males (Fig. 2), although this may have been due to contamination of the female organs with ovarian tissue during dissection.

Time-course studies on aromatase activity were carried out using GAMs from female hatchlings that had been incubated at 30°C. Aromatisation of tritiated steroid substrate by these GAMs was linear up to 9 h under the conditions used here (Fig. 3), validating the 6-h incubation time chosen for routine assays.

To examine the direct effect of assay temperature on aromatase activity, GAMs from stage-21 and stage-25 embryos were incubated with steroid substrate at 30°C, 33°C or 36°C. For stage-21 embryos, incubated at either 30°C or 33°C, aromatase activity in the GAM was very low and not significantly different when assayed at 30°C or 33°C (ANOVA; P>0.05; Fig. 4A). However, for embryos from both incubation temperatures, aromatase activity was significantly reduced when assays were carried out at 36°C. Similar results were obtained when the direct effect of temperature was examined in older embryos, in which clear sex differences in aromatase activity were apparent (stage 25). Aromatase activity in the GAM was high in embryos incubated at 30°C (females) and low in embryos incubated at 33°C (males) when assayed at either 30°C or 33°C (Fig. 4B). Aromatase activity was again inhibited in both sexes when assays were performed at 36°C (Fig. 4B).

Radioimmunoassays (RIAs) carried out on extracted media following the 6-h aromatase assays confirmed higher aromatase activity in the GAMs of female embryo-
Fig. 6 a–f. Development of embryonic alligator gonads at 33°C (a–c) and 30°C (d–f). All micrographs are of 1-μm longitudinal resin sections. a Stage-21/33°C gonad showing a well-defined cortex (C) and underlying medulla (M); insert, higher magnification of the medulla, showing the first signs of the onset of testis differentiation. Small numbers of enlarged pre-Sertoli cells are present in swollen medullary cords (arrows). b Stage-23/33°C gonad showing pre-Sertoli cells differentiating into Sertoli cells as seminiferous cords become organised in the medulla (arrows). The cortex (C) is still well developed. c Stage-25/33°C gonad (testis) showing well-developed seminiferous cords (e.g., S) and reduced cortex (arrows). d Stage-21/30°C gonad showing medulla (M) and cortex (C). The gonad adjoins the mesonephric kidney (Ms); insert, high-power view of the medulla. Most cordal cells are small and irregular in shape (e.g., arrows), compare with a, insert. e Stage-23/30°C gonad showing germ cell proliferation in the cortex (arrows). The medulla (M) shows signs of regression, marked by the fragmentation of medullary cords and the presence of many dark interstitial cells. f Stage-25/30°C gonad (ovary) showing a thickened cortex (C) and regressing medulla (M); bars in a, c, d, and f, 50 μm; bars in b and e, 20 μm; bars in inserts, 10 μm; toluidine blue staining.
Fig. 7a–e. Gonad from stage-28 (hatchling) alligator incubated at 34.5°C. a Transverse section through the anterior pole of the gonad, showing ovarian characteristics. The cortex (C) is well developed, although with few germ cells, and the medulla (M) is fragmented. b Higher magnification of the medulla shown in a. The medullary cords (Mc) are unorganised and separated by extensive lacunae (L). c Transverse section through the posterior pole of the same gonad, just anterior to the medullary rest. The cortex (C) is still well developed, but the medulla (M) is not fragmented. d Higher magnification of the medulla shown in c. The medullary cords (Mc) are organised into structures resembling seminiferous cords, with large polarised cells. The cortex (C) is distinct. e Electron micrograph of one enlarged medullary cord cell seen in d. The polarised cell has ultrastructural characteristics of a Sertoli cell, including a large ovoid nucleus (N) with its long axis perpendicular to the basement membrane (BM), prominent nucleolus (arrow) and apical cytoplasm (Cy). Extensive cytoplasmic processes (Cp), typical of seminiferous cords, are also present; bars in a and c, 50 μm; bars in b and d, 20 μm; bar in e, 5 μm.
os than in males. For embryos incubated at both 30° C (low temperature females) and 34.5° C (high temperature females) estradiol synthesis by the GAM in the presence of tritiated androstenedione increased over development at the stages tested; from 0.5–1 pg/GAM at stage 23–22 to 25 pg/GAM at stage 28 (Fig. 5). In contrast, very low levels of estradiol were measured after aromatase assay of male GAMS over stages 23–28 (Fig. 5).

Gonadal histology

At both 33° and 30° C, embryonic gonads were morphologically undifferentiated at stage 19, when aromatase activity in the GAM was detectable but extremely low and similar at both temperatures. By stage 21 at 33° (male-producing), the gonad had a well developed cortex and an underlying medulla comprising loosely organised medullary cords together with darkly staining interstitial cells (Fig. 6a). High magnification of the gonadal medulla showed the earliest signs of the onset of testis differentiation in these embryos, characterised by the appearance of small numbers of enlarged pre-Sertoli cells within medullary cords (Fig. 6a, insert). By stage 23 at 33°, enlarged pre-Sertoli cells were widespread and they were differentiating into Sertoli cells as seminiferous cords became organised in the medulla of the gonad (Fig. 6b). At stage 25 at 33°, differentiated testes were present, with well-developed seminiferous cords and a reduced cortex (Fig. 6c).

The gonads of stage-21 embryos incubated at 30° (female-producing) had a well-defined cortex and medulla, as at 33° C (Fig. 6d). However, gonads at 30° did not show the early signs of organisation in the medulla as seen in presumptive male embryos; most cells in the medullary cords were smaller, with relatively less cytoplasm and smaller nuclei (Fig. 6d, insert). By stage 23 at 30°, when aromatase activity was still low and a low level of in vitro estradiol synthesis was detected by RIA, the gonad showed signs of ovarian differentiation. Large pale germ cells and darker somatic cells were proliferating in the cortex, while the medullary cords were becoming more loosely organised (fragmented) than at earlier stages (Fig. 6e). By stage 25 at 30°, when aromatase activity in the GAM was high and synthesis of estradiol in vitro was elevated, all gonads examined were ovaries, characterised by a thickened cortex with meiotic germ cells and an extensively fragmented medulla (Fig. 6f).

Gonadal development in female embryos incubated at 34.5° C appeared similar to that of low temperature females at stages 19, 21, 23 and 25, with cortical proliferation and regression of the medulla also evident at stage 23. However, close examination of the ovaries of three female hatchlings (stage 28) incubated at 34.5° C revealed some apparent seminiferous cord development towards the caudal pole of the gonad. Anteriorly, the gonads had a typical ovarian structure. The cortex was well developed (although with few germ cells in those sections examined), and the medulla was fragmented (Fig. 7a). In the medulla, epithelial cords were loosely arranged, and lacunae were present within cords (Fig. 7b). At the posterior pole, just anterior to the so-called medullary rest (where regression of the medulla and proliferation of the cortex do not occur), the cortex was well developed, while the medullary cords were not fragmented. Instead, the cords were organised into rounded structures (Fig. 7c) that strongly resembled seminiferous cords (Fig. 7d). Indeed, the cells within these cords showed ultrastructural characteristics of Sertoli cells. The typical cell was elongated and polarised, with a basal nucleus and apical cytoplasm. The long axis of the nucleus was oriented perpendicular to the basement membrane, and the nucleolus was prominent (Fig. 7e).

Discussion

The results of this study indicate that in alligator embryos aromatase activity in the GAM increases during development only at female-producing temperatures. This increased aromatase activity accompanies morphological differentiation of the ovary. The level of aromatase activity found in this study, and the pattern of increase in female alligator GAMS, is similar to that previously reported for female crocodile (Crocodylus porosus) embryos [42].

It has been hypothesised that aromatase synthesis or activity may be thermosensitive in embryonic reptiles with TSD. This would provide a mechanism whereby temperature might mediate estrogen synthesis, which in turn influences gonadal sex differentiation ([11, 10, 12], reviewed in [36]). In this study, temperature did not directly influence aromatase activity per se when assays were carried out at 30° and 33° C (Fig. 4). For aromatase to be thermosensitive, temperature must directly or indirectly regulate aromatase synthesis (or breakdown) in embryonic alligator GAMS. Temperature-regulation of aromatase synthesis has been proposed for Emys orbicularis (a turtle with TSD), where gonadal aromatase activity also increases during female development but not during male development [10, 11]. The inhibition of aromatase activity at high assay temperature (36° C) in A. mississippiensis is consistent with previous results reported for C. porosus embryos, where high assay temperature (35°–37° C) also inhibited aromatase activity [42]. This inhibition may represent a direct effect of temperature on enzyme kinetics, or an indirect effect upon the GAM or the culture medium used in the assay. In any case, such direct temperature inhibition of aromatase activity is not relevant to sex determination under natural conditions; constant temperatures above 35° C are incompatible with survival in embryonic alligators [30].

Since increased aromatase activity was not detectable until after the TSP for sex determination at 30° C (Fig. 1), aromatase may be a downstream component of the female-determining pathway in alligator embryos. Incubation temperature may control regulatory genes which in turn control the differentiation of somatic steroidogenic cells and hence aromatase production. In an-
other crocodilian, *C. porosus*, increased aromatase activity in the GAM of female embryos was also undetectable until after the TSP [42]. Furthermore, in both *A. mississippiensis* and *C. porosus*, morphological differentiation of the ovary appears to begin towards the end of the TSP [41, 42]. Lance and Bogart [29] have detected estradiol in the plasma of alligator embryos throughout incubation at male- and female-producing temperatures, but no clear sex differences were apparent. Altogether, these observations suggest that unidentified female-determining genes may be activated prior to morphological differentiation of the ovary and increased estrogen synthesis. Thus, aromatase may be indirectly controlled by temperature. In contrast, gonadal aromatase activity does increase during the TSP in embryos of the turtle, *Emys orbicularis*, incubated at the female-producing temperature [10], providing evidence that aromatase lies close to the beginning of the ovary-determining pathway.

However, as in previous studies [10, 12], this study did detect low levels of aromatase enzyme activity throughout the TSP in both presumptive sexes. We cannot exclude the possibility that small, local differences in enzyme levels may exist at male- and female-producing temperatures, differences that were not detected by the whole organ assay technique used here. Indeed, a comparison of Fig. 1 and 5 shows that aromatase activity and estradiol synthesis in vitro (in the same tissues) were not always correlated. Although there were no detectable sex differences in aromatase activity at stage 23, for example (the end of the TSP) (Fig. 1), small but significant sex differences in estradiol synthesis in vitro were detected (Fig. 5). Other studies have used microsomal preparations rather than whole tissues to measure aromatase activity [25] and this might be a more sensitive technique. However, previous studies using the turtle *E. orbicularis* have relied exclusively on the whole organ method [10, 11, 12] and this seemed sufficient to reveal (albeit very small) sex differences in aromatase activity.

The most likely site of aromatase activity in the GAM of female embryos is the gonad, as suggested by the high levels of enzyme activity in isolated hatchling ovaries compared to those in the adrenal-mesonephros (Fig. 2). The cell lineage in the developing ovary responsible for enhanced aromatase activity is, however, not known. It seems unlikely to be the germ cell lineage, since, in bird embryos, female gonads can undergo somatic differentiation and synthesise estrogens when the germ cell population has been significantly reduced [33]. The somatic (pre-follicular) cells of the cortex, medullary cord cells or interstitial cells of the medulla are alternative sources. Pre-follicular cells begin proliferating and the medullary cords show signs of regression between stages 22 and 23 (i.e., towards the end of the TSP) in alligator embryos incubated at 30°C [41], which corresponds with the onset of increased detectable estradiol production in female GAMs observed here (Fig. 5). We have recently attempted immunohistochemical localisation of aromatase in embryonic alligator gonads but have been unable to detect the enzyme, using antibodies directed against either the mammalian or the bird protein.

This study is the first to describe aromatase enzyme activity throughout female development at both high and low egg incubation temperatures in a reptile with TSD. Since aromatase activity increased in the GAM of female embryos incubated at both 30° and 34.5°C, it might be argued that aromatase production is only temperature-dependent by virtue of its inhibition at the male-producing temperature (33°C). Enhanced aromatase activity in the GAM may occur constitutively during alligator embryogenesis, playing a role in ovary differentiation, unless blocked by a factor activated at a critical (male-determining) temperature (or small temperature range). This factor would directly or indirectly divert development towards the male pathway. For alligators and other crocodilians, the factor would be variably active at 32° to 33°C (male-producing for all species) [30]; on either side of this temperature window, production of the factor may be suboptimal or absent, allowing aromatase synthesis, ovary differentiation and female development. (In this context, no reptilian species has yet been shown to exhibit a male-female-male pattern of TSD).

Evidence from this and other studies indicate that increasing aromatase activity (and hence estrogen synthesis) is correlated with proliferation of the cortex and regression of the medullary cords during ovary development in species with TSD [11, 28, 56]. Lance and Bogart [28] found that administration of a non-steroidal aromatase inhibitor, Ciba Geigy 16949A (fadrozole) disrupted ovarian differentiation in alligator embryos incubated at 30°C. The cortex did not show normal germ cell proliferation and/or medullary cords did not regress. However, the gonads did not show any signs of testis differentiation. In genetically female chicken embryos, administration of the same aromatase inhibitor has been shown to induce testis differentiation [15].

Aromatase enzyme activity may be inhibited by anti-Müllerian hormone (AMH) in alligator embryos incubated at the male-producing temperature. Bovine AMH can inhibit aromatase activity in the ovaries of embryonic rats [49], and in the embryonic ovaries of a turtle with TSD [13]. However, evidence from mammals indicates that AMH is located downstream in the sex-determining cascade [34]. Furthermore, studies on Müllerian duct regression in male alligator embryos suggest that the hormone is active towards the end of incubation in male embryos [3]. Therefore, although AMH may negatively regulate aromatase in male alligator embryos later in development, it may not be the only putative inhibitor of the enzyme and other factors may be involved earlier in embryogenesis.

The development of some apparent seminiferous cords and Sertoli cells in the ovaries of the high-temperature females examined indicates that both male- and female-specific tissue can develop within the same gonad in alligator embryos. This may be related to the fact that 34.5°C is close to the exclusively male-producing temperature of 33°C (indeed, small numbers of males are produced at 34.5°C). Female-producing temperatures close
to the male-producing temperature may allow some development of seminiferous cords, particularly near the posterior pole of the gonad, where medullary cord regression and cortex proliferation do not normally occur in female alligator embryos (the so-called "medullary rest" described by Forbes [20]). It would be interesting to examine the gonads of female embryos produced at other temperatures close to the male-producing temperature (e.g., 32°C, which yields mainly males but some females [30]).

The widespread observation that exogenous estrogens can "override" TSD (and GSD; reviewed in [38, 53, 54]) may be explained by the fact that both presumptive sexes have the capacity to respond to estrogens. Uptake of radiolabelled estradiol by embryonic crocodile gonads [44] and estrogen receptor in situ hybridisation studies in turtle embryos [7] support this idea. Indeed, the fact that exogenous estrogens can induce ovary differentiation at male-producing temperatures implies that pivotal events downstream of estrogen production in the female sex-determining pathway are not thermosensitive (at female vs. male temperatures). The focus is therefore shifting to the (endogenous) synthesis of estrogens as the thermosensitive mechanism involved in TSD. However, although aromatase inhibitors can induce male development at certain intermediate incubation temperatures in species with TSD [55], this does not necessarily prove that aromatase is the initial signal for female development. (An example from human sex determination may be pertinent. Many human females apparently carry a normal copy of the SRY gene, which signals testis differentiation. These individuals are presumably sex-reversed due to a mutation downstream in the testis-determining pathway [37]). For species with TSD, the exact role of aromatase in the sex-determining process will be clarified by expression analysis of the gene during embryogenesis [25].

In conclusion, the data presented here show that ovary development in alligator embryos, at both high and low incubation temperatures, is accompanied by increased aromatase enzyme activity (and estradiol synthesis in vitro). Proliferation of the gonadal cortex and regression of the medulla during ovary formation may depend upon this enhanced estrogen production. However, it is unclear whether enhanced aromatase activity is the thermosensitive trigger for ovarian differentiation or whether it lies further downstream in the female developmental pathway. The lack of detectable sex differences in aromatase activity until after the TSP, that is, until after sex has been determined, suggest that aromatase may be a downstream component of the female-determining cascade in alligators.

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