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Spatiotemporal Expression of Aromatase (CYP19) During Gonadal Sex Differentiation in the Oviparous Lizard *Calotes versicolor*

Bharati S. Khodnapur[1], Robert S. Nindi[1], Rahul M. Handi[1], Jyoti F. Hiremath[1], Santosh K. Dasari[2], Polani B. Seshagiri[2] and Laxmi S. Inamdar[1]*

[1]Molecular Endocrinology, Reproduction and Development Laboratory, Department of Zoology, Karnatak University, Dharwad – 580003, Karnataka, India; ls_doddamani@yahoo.com
[2]Molecular Reproduction, Development and Genetics Division, Indian Institute of Science, Bangalore – 560012, Karnataka, India

Abstract

Steroidal sex hormones play critical rolest during gonadal sex differentiation and development. Among the sex steroids, estradiol acts at the central level in the sexual differentiation of many reptilian species exhibiting temperature-dependent sex determination (TSD). In recent years, the growing amount of information has highlighted the importance of aromatase (CYP19) in irreversibly catalyzing conversion of androgens into estrogens by aromatization during ovarian morphogenesis. To elucidate the involvement of aromatase in this process we investigated the ontogenic and cellular expression of aromatase in the embryos of the lizard *Calotes versicolor* that exhibits a unique pattern of TSD. The eggs of this lizard were incubated at 31.5±0.5°C which is 100% Female-Producing Temperature (FPT). The torso of embryos containing Adrenal-Kidney-Gonadal (AKG) complex was collected during different stages of development and subjected to Western blotting and immunohistochemical analysis. Western blot analysis revealed a single immunoreactive protein at 58 kDa for aromatase in the total protein extracts of AKG complex during early Thermo-Sensitive Period (TSP) suggesting early action of aromatase. A very weak but specific immunoexpression of aromatase noticed in the cytoplasm of adrenocortical cells, mesonephric kidney tubules (stage 28) and medullary region of the gonad (stage 30) during early TSP suggests early onset of aromatase activity which in turn indicates that these are the sites of estrogen biosynthesis. Further, a remarkable increase in the intensity of immunoreaction for aromatase during late TSP and gonadal differentiation stage shows it's up-regulation and estrogen biosynthesis resulting in ovary differentiation. It is inferred that high expression level of aromatase at FPT is associated with proliferation of cortex facilitating differentiation of ovary in *C. versicolor*.

Keywords: Aromatase, Immunohistochemistry, Lizard, Sex Differentiation, Temperature-Dependent Sex Determination, Western Blotting

Abbreviations: AKG: Adrenal-kidney-gonadal complex; CYP19: Cytochrome P450 aromatase enzyme; ERα: Estrogen Receptor alpha; FMFM: Female-Male-Female-Male Pattern of TSD; FPT: Female-producing temperature; MPT: Male –producing temperature; PBS: Phosphate Buffer saline; PVDF membrane: Polyvinylidene Flouride membrane; TBS: Tris Buffered saline; TBS-T: Tris Buffered saline +Tween 20; TSD: Temperature-dependent sex determination; TSP: Thermo-sensitive period.

*Author for correspondence

1. Introduction

In the vertebrates, estradiol-17β has the potential to regulate, in endocrine/autocrine/paracrine manner, a number of different cell types during growth, differentiation and functions of female and male reproductive tissues[1-3]. Several reptilian species, inclusive of all crocodiles, a majority of turtles and a few lizards, exhibit TSD wherein sexual differentiation of the gonad is sensitive to temperature during a critical period of embryonic development called TSP. It has been reported that during TSP gonadal aromatase activity and estrogen biosynthesis in the gonad depend on the incubation temperature of the egg; temperature-based differences in aromatase expression may be a critical step in ovarian determination[4-7]. Consequently, in such TSD-exhibiting species, the estradiol appears to act centrally in sexual differentiation, and aromatase plays an important role in this process. However, the source of estrogen in the reptilian embryos is still unclear.

It has been established that transformation of androgens into estrogens occurs by aromatization. It involves loss of C-19 methyl group and aromatization of ring A which is catalyzed by the key enzyme complex cytochrome P-450 aromatase[8]. Aromatase is cued by environmental temperature in the species exhibiting TSD.

The Indian oviparous lizard *Calotes versicolor* lacks heteromorphic sex chromosomes[9]. It is an interesting model to study sex determination and differentiation as it exhibits a novel FMFM pattern of TSD i.e., the incubation of eggs at 23.5 ± 0.5 and 31.5 ± 0.5°C produces 100% female hatchlings while incubation of eggs at 25.5 ± 0.5 and 34 ± 0.5°C produces 100% male hatchlings[10]. A recent report from our laboratory on the adrenal-kidney-gonadal expression of ERα in the embryos incubated at female-producing temperature (FPT-31.5 ± 0.5°C) revealed that the onset of gonadal estrogen activity coincides with sexual differentiation of gonad which in turn suggests that the estrogen signaling is vital for ovary determination pathway[11].

Since estrogen is a critical endogenous factor during the early stages of ovarian differentiation in TSD reptiles, it is imperative to decipher the possible temperature-specific differences in embryonic aromatase expression as this enzyme irreversibly catalyzes metabolism of androgens into estrogens, which in turn contributes to the gonadal steroid hormone milieu. Therefore, the present study was undertaken to elucidate the ontogenic and cellular expression of aromatase and the role of estrogen during gonadal sex diferentiation through immunolocalization analysis in *C. versicolor* at FPT of 31.5±0.5°C. Since Western blotting is known to be sensitive for detection of small amounts of proteins the same was performed along with immunohistochemistry.

2. Objectives

1. To investigate the ontogeny and expression analysis of aromatase before, during and after sex determination and ovarian differentiation in the lizard *Calotes versicolor*.
2. To explore the cellular localization of aromatase in the embryonic AKG complex of this lizard.

3. Materials and Methods

3.1 The Animal and Developmental Stages

C. versicolor, a polyautochronic, multiclutched lizard, exhibits an extended breeding phase (May to October) and retains the eggs in the oviduct for a period as may be determined by environmental factors, especially soil moisture and rain fall. The developmental stages of eggs during oviductal retention as well as after laying were identified according to Muthukkaruppan *et al.*[12] and Akbarsha[13]. The development of embryos from stage 1 to stage 26 takes place when the eggs are still in the oviduct of the mother. Oviposition occurs at embryonic stage 27 and hatching takes place at stage 42. The interval between two successive stages is approximately 2-3 days long during the earlier (stages 27-35) and 4-5 days long during the later (stages 36-42) part of the development, and it normally takes 60-75 days for hatching. Collection of eggs and incubation methods have already been described elsewhere[10,14-16] . All the experiments were conducted in accordance with the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and as approved by the Institutional Animal Ethical Committee [(IAEC), No. 639/GO/02/a/CPCSEA] of the Karnatak University, Dharwad, Karnataka, India.

3.2 Collection and Incubation of Eggs

In all, 20 clutches of eggs (n=320) from twenty gravid females, were obtained during the breeding season and

randomly assigned and incubated in the incubator at the female-producing temperature of 31.5±0.5°C. Care was exercised to ensure that fluctuation in the incubation temperature did not exceed 0.5°C with a relative humidity of 62%. Other incubation methods have already been described elsewhere[10]. The eggs were incubated from oviposition (stage 27) to hatching (stage 42). Developmental progress was monitored regularly and the stages of embryonic development were determined as per the criteria previously described for *C. versicolor*[12,13] .

The hemipenes that can be seen clearly in male embryos during the embryonic period gets resorbed in one-day-old male hatchlings. Hence, the cloacal sexing was performed prior to experimentation in order to distinguish the sex of the embryo[17]. Reliability of sex was also verified by gonadal histology and the presence of secondary sexual characteristics.

3.3 Preparation of Protein Extract

The embryos (n=10) were dissected in cold PBS (pH-7.4), the torso containing adrenal, kidney and gonads (AKG) was excised and homogenized in RIPA buffer [50 mM Tris-HCl (pH 8.0), with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, Sigma-Aldrich, St. Louis, MO, USA] and clarified by centrifugation at 25,000 x *g* at 4°C. The supernatant was aliquoted and stored at -20°C until use. Protein concentrations were determined using Amido Black B (at 630 nm, ELISA Microplate Reader with Gen5 software, BioTek, USA).

3.4 Western Blotting

The proteins (40 µg) were denatured by being boiled for 5 min in a hot water bath prior to electrophoresis. The supernatant protein samples were fractionated by 12% SDS-PAGE under reducing conditions and transferred onto PVDF membrane (Immobilon Millipore, Bedford, MA, USA). Complete transfer was assessed using pre-stained protein standards (Fermentas Life Sciences, USA). The membranes were blocked with 5% non-fat powdered milk in PBS (25 mM Tris, pH 7.4; 200 mM NaCl; 0.5% Triton X-100, TBS/T) for 2 hr at room temperature. Then, they were incubated at 4°C overnight with rabbit anti-aromatase polyclonal antibody [(1:100 dilutions; CYP19 (H-300)-a rabbit polyclonal antibody raised against amino acids 209-503 mapping at the C-terminus of CYP19 of human origin) sc-30086, Santa Cruz Biotechnology, USA]. After washing with TBS-T and TBS, the membranes were incubated with the Horseradish peroxidase-conjugated (HRP) secondary antibody (1:3000 dilution) for 60 min (at room temperature) and the reaction was detected using an Enhanced Chemi-Luminescence (ECL) system (Image Quant, LAS 4000 and GE, USA). The membranes were then washed twice in TBS-T for 10 min each before being re-probed with anti-actin antibody (diluted in TBS-T) which served as internal control. All results were expressed relative to β-actin. Band intensities were measured using ImageJ software and aromatase/β-actin ratios were determined.

3.5 Immunohistochemistry

The torso of embryos (n=5) containing AKG complex was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 µm on a rotary microtome (Leica RM2025). Antigen retrieval for aromatase was performed by placing slides in trisodium citrate buffer (pH=6.0, C3674 Sigma-Aldrich, St. Louis, MO, USA) followed by washing with PBS. The endogenous peroxidase was inactivated with 3% hydrogen peroxide (H_2O_2). Nonspecific binding was blocked by 5% BSA (A3059, Sigma-Aldrich, St. Louis, MO, USA). Subsequently, sections were incubated with rabbit anti-aromatase polyclonal antibody (H-300, sc-30086; 1:100; Santa Cruz Biotechnology, Dallas, Texas, USA) at 4°C overnight. As a negative control, primary antibodies were replaced with antibody diluent (1% BSA). After three steps of washing with PBS and PBS-T, the sections were incubated with the Goat anti-rabbit IgG conjugated to HRP (sc-2004-secondary antibody; 1:200, Santa Cruz Biotechnology, Dallas, Texas, USA). Staining was visualized with 3, 3'-diaminobenzidine tetra-hydrochloride hydrate (DAB; D5637, Sigma-Aldrich, St. Louis, MO, USA). Finally, sections were counterstained with Harris-hematoxylin. Images were acquired using Nikon Eclipse 80i with ACT-2U software (Nikon Corporation, Tokyo, Japan).

4. Results

4.1 Western Blotting

Western blot analysis was carried out during different stages of development to verify the antibody specificity for aromatase protein content in the AKG complex of *C. versicolor* embryos incubated at FPT. The aromatase antibody recognized a single protein band with apparent molecular weight 58 kDa in the total protein extract of AKG complex (Figure 1A-C). The onset of AKG

immunoexpression of aromatase (trace activity) at 58 kDa was initiated at stage 28 i.e., during early Thermosensitive Period (Pre-TSP). The duration of TSP extends between the embryonic stages 30 and 33. An increase in its expression was observed during the late TSP. Subsequently, the AKG expression of aromatase revealed an increase in the intensity pattern of immunoreaction during and after gonadal differentiation (stages 34 to 37) which was sustained thereafter (Figure 1B). However, immunoexpression of aromatase was observed to be weak/in traces at hatching (Figure 1C). Overall the AKG expression of aromatase at FPT was specific, persisted throughout the developmental period and showed a tendency to increase from stage 33 to 37 (Figure 1A&B).

4.2 Immunoreactivity for Aromatase

In order to determine precisely the specific cellular localization of aromatase we carried out immunohistochemical study. Though the AKG expression of aromatase was studied for all the developmental stages, emphasis was given for the following four important phases of development.

4.3 Pre-TSP (Stages 28-29) and TSP (Stages 30-33)

During these stages, the AKG complex consisted of the gonad appearing as genital ridge having primordial germ cells, a few adrenocortical cells and mesonephric kidney tubules. Onset of expression of aromatase was noticed during early TSP. Cytoplasmic expression of aromatase was observed to be very faint in the adrenocortical cells (Figure 2A), mesonephric kidney tubules at stage 28 (Figure 2B), and medulla of gonadal anlagen (stage 30) of the AKG complex (Figure 2C).

During late TSP (at stage 33) expression of aromatase was clearly evident in the medullary region of the gonad and also in the oogonia of the cortex (Figure 3A). Prominent immunoexpression of aromatase was noticed in the cytoplasm of adrenocortical cells as well.

4.4 Gonadal Differentiation (Stage 34)

During gonadal differentiation an intense immunoexpression of aromatase was found essentially confined to cytoplasm of medullary cells and oocytes of the cortex (Figure 3B&C). Furthermore, a prominent cytoplasmic immunoexpression of aromatase in the adrenocortical cells was evident during gonadal sex differentiation stage (Figure 3D).

4.5 Post-Gonadal Differentiation (Stages 35-38)

A noticeable difference in the intensity and pattern of immunoexpression was observed in the AKG complex. An apparent increase in the immunoexpression of aromatase was discerned in the medullary cells as well as oocytes (cortex region) of the ovary during post-ovarian differentiation phase (Figure 4A). Likewise, the adrenocotical cells showed clear cytoplasmic expression of aromatase. No specific staining was noticed in the control AKG complex when neither the primary nor secondary antibodies were included in the staining run (Figure 4B).

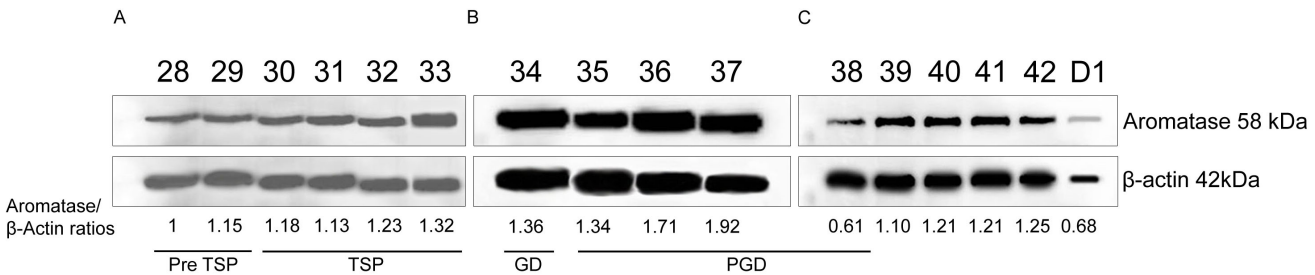

Figure 1. Immunoblot expression of aromatase (CYP19) in the adrenal- kidney-gonadal complex (AKG) of *Calotes versicolor* embryos (Embryonic stages 28 to one-day-old hatchling) incubated at female-producing temperature (FPT - 31.5±0.5°C). Immunoblot reveals the onset of aromatase in the AKG complex at stage 28. **A.** AKG expression of aromatase during the embryonic stages 28 to 33 (Pre-TSP and TSP stages); **B.** AKG expression of aromatase during the embryonic stages 34 to 37; **C.** AKG expression of aromatase during the embryonic stages 38 to D1 in C. *versicolor*. β-actin was used as control. Pre-TSP - Pre-Thermo-sensitive period; TSP - Thermo-sensitive period; GD - Gonadal differentiation; PGD - Post gonadal differentiation; D1 - One-day-old hatchling.

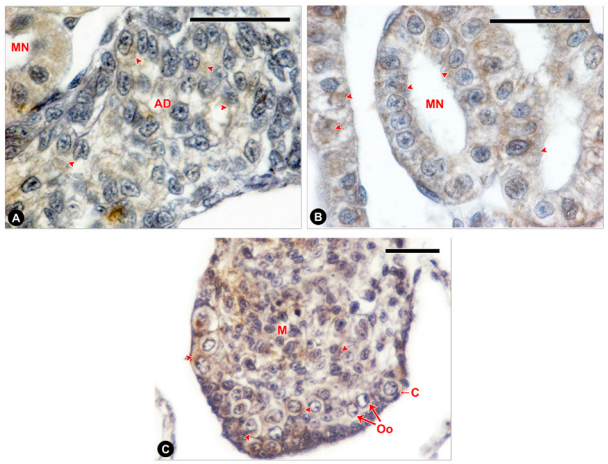

Figure 2. Immunolocalization of aromatase (CYP19) in the AKG of C. *versicolor* embryos during pre-TSP and TSP stages incubated at FPT as determined by immunohistochemistry. **A.** T.S. of stage 28 embryo (early TSP) showing onset of cytoplasmic expression (traces) of aromatase (arrows) in the adrenocortical cells. AD - Adrenocortical cells. MN- Mesonephros Scale bar - 10 µm. **B.** T.S. of stage 28 embryo revealing aromatase expression (arrows) in the mesonephric kidney tubules (MN). Scale bar - 10 µm **C.** T.S. of stage 30 embryo showing onset of cytoplasmic expression of aromatase (arrows) in both cortical (C) and medullary (M) regions of the gonad. Scale bar - 30 µm.

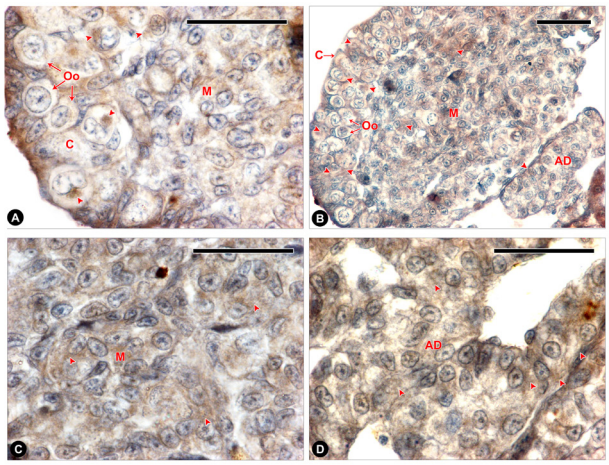

Figure 3. Immunolocalization of aromatase (CYP19) in the adrenocortical cells and ovary of C. *versicolor* embryos incubated at FPT (embryonic stages 33 and 34). **A.** T.S. of stage 33 embryo (late TSP) revealing high expression of aromatase (arrows) in the ovary. M - Medulla; C - Cortex; Oo - Oogonia. Scale bar - 10 µm **B.** T.S. of stage 34 embryo (gonadal differentiation stage) revealing an enhanced cytoplasmic expression of aromatase (arrows) in the ovary. M - Medulla; C - Cortex; Oo - Oogonia; AD - Adrenocortical cells. Scale bar - 30 µm **C.** Magnified view of T.S. of stage 34 embryo showing intense immunostaining of aromatase (arrows) in the medullary part of the ovary. M - Medulla. Scale bar - 10 µm **D.** T.S. of stage 34 embryo revealing a prominent cytoplamic immunostaining of aromatase (arrows) in the adrenocortical cells. AD - Adrenocortical cells. Scale bar - 10 µm.

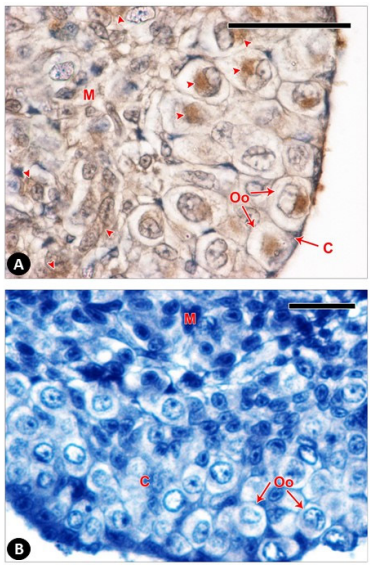

Figure 4. Immunolocalization of aromatase (CYP19) in the ovary of C. *versicolor* embryos incubated at FPT (embryonic stage 37). **A.** T.S. of stage 37 embryo showing an intense immunostaining of aromatase (arrows) in the ovary. C - Cortex; M - Medulla; Oo - Oocytes. Scale bar - 10 µm **B.** T.S. of stage 37 control embryo showing ovary (without primary antibody). Note the cortex (C) consisting of many oocytes (Oo). M – Medulla. Scale bar - 30 µm.

5. Discussion

In TSD reptiles, the aromatase activity and estrogen biosynthesis play an integral role in the early steps of ovarian differentiation pathway.

In the present study, Western blot analysis was carried out to confirm the antibody specificity. The aromatase antibody recognized a single reactive protein band with apparent molecular weight ~58 kDa in the total protein extract of AKG complex of *C. versicolor* embryos incubated at FPT. The onset of AKG expression of aromatase at 58 kDa occurs at stage 28, i.e., Pre-TSP, suggesting an early role to aromatase. An increase in the intensity pattern of immunoreaction is observed during late TSP and post-gonadal differentiation stages that is sustained during later stages of development. Enhanced expression of aromatase in the AKG complex indicates its up-regulation leading to increased biosynthesis of estrogen as this enzyme is key mediator of endogenous production of estrogen. In another study, the immunoblot of aromatase showed a single band of 58 kDa in the AKG complex of this lizard embryos incubated at MPT (25.5 ± 0.5°C). Nevertheless, the intensity of the signal was not uniform during late TSP and it declined drastically during testis differentiation[18]. The Western blot of embryonic testis of the turtle *T. scripta* yielded two immunoreactive bands for aromatase at 48 and 60 kDa[19].

The very weak but specific cytoplasmic immunolocalization of aromatase in the embryonic adrenocortical cells, and mesonephric kidney tubules during early TSP (stage 28) and medullary region of the gonad (stage 30) suggests the onset of aromatase activity, thus revealing the early action of aromatase. The present investigation also indicates that the female-producing temperature of 31.5±0.5°C favors aromatase activity in this lizard. Induction of this enzyme during early TSP is necessary as this is the terminal enzyme complex which executes three successive monooxygenations on androstenedione or testosterone resulting in the formation of estrone or estradiol. Further, the onset of immunoexpression of aromatase (present study) and ERα[11] in the adrenocortical cells precedes expression in the gonad. Hence, the steroidogenic potency of adrenocortical cells acting as initial source of steroid substrate (in a paracrine manner) for biosynthesis of estradiol may be considered, which further supports our earlier reports[14-16]. Additionally, our previous experiment on the AKG sex steroid levels [Estradiol (E_{2}) and Testosterone (T)] reveals that the initial levels of testosterone (at oviposition-stage 27 and during pre-TSP-stage 28) are significantly higher than those of estradiol at both FPT and MPT. Consequently, the available testosterone, considered as prohormone for estradiol, may also be metabolized locally (adrenaocortical cells) into estrogen through aromatization at FPT. Nevertheless, the level of estradiol increased exponentially during late TSP and ovarian differentiation[20]. Therefore, the key role played by aromatase in conversion of androgens to estrogens at FPT needs to be considered. Moreover, it is to be noted that the expression of ERα in our previous study[11] and that of aromatase (present study) are seen in the same cells (AKG) strongly supporting our contention that essentially these are the sites of estrogen biosynthesis during gonadal sex differentiation. Therefore, the observed results of the AKG expression of aromatase suggest that the medullary region and oocytes (cortex) of ovary, adrenocortical cells and mesonephric kidney tubules are steroidogenically active sites for biosynthesis of estrogen during gonadal differentiation

Further, a predominant expression in the intensity and pattern of the immunoreaction of aromatase in the adrenocortical cells and medullary cells of the gonad during late TSP and gonadal differentiation stages suggest its up-regulation at FPT. This increased expression of aromatase suggests an enhanced biosynthesis of estradiol which may act in an endocrine and/or paracrine mode to induce proliferation of the cortex thus facilitating ovarian differentiation. A recent report from our laboratory on the AKG expression of ERα in the embryos incubated at FPT reveals that the onset of gonadal estrogen activity coincides with sexual differentiation of the gonad suggesting that the estrogen signaling is vital for ovarian determination pathway[11]. We have also noted that an apparent surge in endogenous estradiol coincides with the first indication of ovarian differentiation at FPT in this lizard[20]. Therefore, increased expression of aromatase at FPT represents its enhanced activity leading to proliferation of cortex thereby promoting ovary differentiation in *C. versicolor*

Therefore, based on the Western blotting and immunohistoshemical study we provide evidence that the aromatase (CYP19) enzyme is up-regulated at FPT during TSP and during ovarian differentiation stages. Our present study supports the statement of Pieau and Dorizzi[5] that incubation temperature during TSP up-regulates (or down-regulates) the aromatase gene, thus leading to production of estrogen (or suppression of estrogen biosynthesis). In the

pond turtle, *Emys orbicularis,* at the beginning of the TSP, the aromatase activity was very low at both MPT and FPT, respectively. It subsequently increased exponentially during late TSP and formed a peak in differentiating ovaries while it remained low in testis[21]. Further, qPCR experiments showed that gonadal aromatase is expressed higher at female- than at male-producing temperatures in *Chelydra serpentina*[22] and in *Trachemys scripta*[23,24]. Isolated gonadal tissue rather than the whole AKG tissue was utilized as target in these studies. Also, in the embryos of *Malaclemys terrapin,* aromatase mRNA in the AKG of females is significantly higher than in males during gonadal differentiation[25]. However, in the American alligator, *Alligator mississippiensis,* gonadal aromatase activity remained low during the early stages of TSP in both the sexes and increased in the late TSP in females only[26,27]. In addition, elevated expression of aromatase was consistent in differentiating ovaries among amphibians and birds while its expression is usually suppressed during development of testes[28-30].

Put together, our study matches with the above-mentioned studies and demonstrates that ovary differentiation in *C. versicolor* (at FPT) is in harmony with increasing aromatase expression and estrogen biosynthesis. Therefore, increased expression of aromatase at FPT represents its enhanced activity which is in synchrony with proliferation of cortex thereby promoting ovary differentiation in *C. versicolor.*

Following are the important findings of the present study:

1. The feeble but specific cytoplasmic expression of aromatase observed in the adrenocortical cells, medullary region of the gonad and mesonephric kidney tubules during early TSP at FPT in *C. versicolor* suggests the onset of aromatase activity thus revealing its early action.
2. A remarkable increase in the intensity of the immunoreaction for aromatase during late TSP and gonadal differentiation stage reveal it is up-regulation and estrogen biosynthesis, thus promoting ovary differentiation.
3. In conclusion, high expression/up-regulation of aromatase in the AKG complex at FPT is associated with proliferation of cortex, resulting in ovary differentiation in *C. versicolor.*

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7. References

1. Clark JH, Schrader WT, O'Malley BW. Mechanism of Action of Steroid Hormones. In: Wilson JD, Foster DW (Ed), Textbook of Endocrinology, Eighth Ed, WB Saunders Company, Philadelphia; 1992. p. 35-90,
2. Sharpe RM. The roles of oestrogen in the male. Trends in Endocrinology and Metabolism. 1998; 9:371-377. https://doi.org/10.1016/S1043-2760(98)00089-7.
3. Carreau S, Hess RA. Oestrogens and spermatogenesis. Philosophical Transactions of Royal Society B. 2010; 365:1517-1535. https://doi.org/10.1098/rstb.2009.0235. PMid:20403867 PMCid:PMC2871919.
4. Crews D, Bergeron JM, Bull JJ, Flores D, Tousignant A, Skipper JK, Wibbels T. Temperature-dependent sex determination in reptiles: Proximate mechanisms, ultimate outcomes, and practical applications. Development and Genetics. 1994; 15:297-312. https://doi.org/10.1002/dvg.1020150310. PMid:8062460.
5. Pieau C, Dorizzi M. Oestrogens and temperature-dependent sex determination in reptiles: All is in the gonads. Journal of Endocrinology. 2004; 181:367-377. https://doi.org/10.1677/joe.0.1810367. PMid:15171684.
6. Lance VA. Is regulation of Aromatase expression in reptiles the key to understanding temperature-dependent sex determination? Journal of Experimental Zoology. 2009; 311A: 314-322. https://doi.org/10.1002/jez.465. PMid:18668631.
7. Nakamura M. The mechanism of sex determination in vertebrates-are sex steroids the key-factor? Journal of Experimental Zoology. 2010; 313A:381-398. https://doi.org/10.1002/jez.616. PMid:20623803.
8. Fisherman J, Goto J. Mechanism of estrogen biosynthesis. Participation of multiple enzyme sites in placental aromatase hydroxylations. Journal of Biologocal Chemistry. 1981; 256:4466-4471. https://doi.org/10.1016/S0021-9258(19)69458-5.
9. Singh L. Study of mitotic and meiotic chromosomes in seven species of Lizards. Proceedings of the Zoological Society. 1974; 27:57-59.
10. Inamdar (Doddamani) LS, Vani V, Seshagiri PB. A tropical oviparous lizard, *Calotes versicolor*, exhibiting a potentially

novel FMFM pattern of temperature-dependent sex determination. Journal of Experimental Zoology. 2012a; 317A:32-46. https://doi.org/10.1002/jez.718. PMid:22021250.
11. Inamdar LS, Khodnapur BS, Nindi RS, Dasari S, Seshagiri PB. Differential expression of estrogen receptor alpha in the embryonic adrenal-kidney-gonadal complex of the oviparous lizard, *Calotes versicolor* (Daud.). General and Comparative Endocrinology. 2015; 220:55-60. https://doi.org/10.1016/j.ygcen.2014.08.003. PMid:25127850.
12. Muthukkaruppan VR, Kanakambika P, Manickavel V, Veeraraghavan K. Analysis of development of the lizard, *Calotes versicolor*. I. Series of normal stages in the embryonic development. Journal of Morphology. 1970; 130:479-489. https://doi.org/10.1002/jmor.1051300407. PMid:5437480.
13. Akbarsha MA. Development of parathyroid glands of the lizard *Calotes versicolor* (Daud In). Zeitschrift für Mikroskopisch-Anatomische Forschung. 1985; 99:204-208.
14. Doddamani LS. Histoenzymological studies on the embryonic and post hatching development of the ovary in the tropical oviparous lizard, *Calotes versicolor*. Journal of Morphology. 1994; 221:1-10. https://doi.org/10.1002/jmor.1052220102. PMid:29865425.
15. Doddamani LS. Development of the adrenal gland in the tropical lizards, *Calotes versicolor*. General and Comparative Endocrinology. 2000; 117:89-102. https://doi.org/10.1006/gcen.1999.7385. PMid:10620426.
16. Doddamani LS. Differentiation and development of testis in the oviparous lizard, *Calotes versicolor*. Journal of Experimental Zoology. 2006; 305A:299-308. https://doi.org/10.1002/jez.a.265. PMid:16432882.
17. Inamdar (Doddamani) LS, Vani V, Advi Rao GM. Dimorphic hemipenis correlates the pathways of gonadal sex differentiation in the lizard *Calotes versicolor* (Daud.). Advances in Zoology. 2012b; 33:3-37.
18. Robert NS, Advi Rao, GM, Inamdar (Doddamani) LS. Adrenal-Kidney-Gonadal expression of aromatase (CYP19) in the male embryos of the Lizard, *Calotes versicolor*- A western blot analysis. Journal of Chemical Biological and Physical Sciences B. 2016; 6:533-539.
19. Gist DH, Bradshaw X, Morrow CMK, Congdon JD, Hess RA. Estrogen response system in the reproductive tract of the male turtle: An immunocytochemical study. General and Comparative Endocrinology. 2007; 151:27-33. https://doi.org/10.1016/j.ygcen.2006.06.012. PMid:16935286.
20. Khodnapur BS, Nindi RS, Inamdar LS. Sex Steroid Hormone Profiles (t & e2) during Sexual Differentiation of a Reptile with Temperature-Dependent Sex Determination. In: International Conference on Bioactive Chemicals for Reproduction and Human Health & 33rd Annual Meeting of The Society for Reproductive Biology and Comparative Endocrinology. Proceedings; 2015. p. 173-174.
21. Desvages GM, Pieau C. Aromatase activity in gonads of turtle embryos as a function of the incubations temperature of eggs. Journal of Steroid Biochemistry and Molecular Biology. 1992; 41:3-8. https://doi.org/10.1016/0960-0760(92)90437-N.
22. Rhen T, Metzger K, Schroeder A, Woodward R. Expression of putative sex-determining genes during the thermo-sensitive period of gonadal development in the snapping turtle, *Chelydra serpentina*. Sexual Development. 2007; 1:255-270. https://doi.org/10.1159/000104775. PMid: 18391536.
23. Ramsey M, Crews D. Adrenal-kidney-gonad complex measurements may not predict gonad-specific changes in gene expression patterns during temperature-dependent sex determination in the red-eared slider turtle (*Trachemys scripta* elegans). Journal of Experimental Zoology. 2007; 307A:463-470. https://doi.org/10.1002/jez.399. PMid:17592622.
24. Ramsey M, Shoemaker C, Crews D. Gonadal expression of Sf1 and aromatase during sex determination in the red-eared slider turtle (*Trachemys scripta*), a reptile with temperature-dependent sex determination. Differentiation. 2007; 75:978-991. https://doi.org/10.1111/j.1432-0436.2007.00182.x. PMid:17490415.
25. Pancharatnam S, Place AR. Temperature-dependent aromatase expression in developing diamondback terrapin (*Malaclemys terrapin*) embryos. Journal of Steroid Biochemistry and Molecular Biology. 1997; 61:3-6. https://doi.org/10.1016/S0960-0760(97)80041-6.
26. Smith CA, Elf PK, Lang JW, Joss JMP. Aromatase activity during gonadal sex differentiation in alligator embryos. Differentiation. 1995; 58:281-290. https://doi.org/10.1046/j.1432-0436.1995.5840281.x.
27. Milnes MR, Roberts RN, Guillette LJ. Effects of incubation temperature and estrogen exposure on aromatase activity in the brain and gonads of embryonic alligators. Environmental Health Perspectives. 2002; 110:393-396. https://doi.org/10.1289/ehp.02110s3393. PMid:12060834 PMCid:PMC1241188.
28. Smith CA, Andrews JE, Sinclair AH. Gonadal sex differentiation in chicken embryos: expression of estrogen receptor and aromatase genes. Journal of Steroid Biochemistry and Molecular Biology. 1997; 60:295-302. https://doi.org/10.1016/S0960-0760(96)00196-3.
29. Kuntz S, Chesnel A, Flament S, Chardard D. Cerebral and gonadal aromatase expressions are differently affected during sex differentiation of Pleurodeles waltl. Journal of Molecular Endocrinology. 2004; 33:717-727. https://doi.org/10.1677/jme.1.01588. PMid:15591030.
30. Sakata N, Tamori Y, Wakahara M. P450 aromatase expression in the temperature-sensitive sexual differentiation of salamander (*Hynobius retardatus*) gonads. International Journal of Developmental Biology. 2005; 49:417-425. https://doi.org/10.1387/ijdb.041916ns. PMid:15968587.