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Letter to the editor Open Access

Role of *Cyp19a1* in the female pathway of a freshwater turtle species (*Mauremys reevesii*) with temperature-dependent sex determination

The molecular mechanisms underpinning temperature-dependent sex determination (TSD) in reptiles have attracted great biological interest for many years. However, which genetic factors are essential for TSD remain elusive, especially regarding female sex determination. Cytochrome P450 family 19 subfamily A member 1 (*Cyp19a1*) encodes the endoplasmic reticulum enzyme aromatase, which participates in the catalytic conversion of androgens to estrogens, and is implicated in sexual differentiation in many species. However, whether *Cyp19a1* plays a critical role in determining gonadal sexual fate in TSD species remains to be elucidated. In the current study, *Cyp19a1* exhibited a temperature-dependent and sexually dimorphic expression pattern, preceding gonadal sex differentiation in a TSD turtle species (*Mauremys reevesii*). Sexual phenotype of the turtles was successfully reversed by aromatase inhibitor treatment at the female-producing temperature (FPT). Furthermore, exogenous estradiol (E2) treatment led to complete male-to-female sex reversal at the male-producing temperature (MPT), accompanied by rapid up-regulation of *Cyp19a1*. Thus, *Cyp19a1* appears to be essential for female sex determination in *M. reevesii*, suggesting a vital role in the female TSD pathway.

In reptiles, the molecular mechanisms underlying TSD have been researched for several decades. Early studies focused on hormone-dependent mechanisms and the cloning and/or expression of certain classical sex-related genes involved in the genetic sex determination (GSD) system (e.g., *Wt1*; *Amh*) in TSD species (Pieau et al., 1999). Later studies demonstrated that several of these genes exhibit temperature-dependent expression patterns during thermo-sensitive periods (TSP), prior to gonadal sex differentiation (Shoemaker et al., 2007). Recent research on the transcriptomes of TSD reptile taxa identified a great number of candidate genes involved in gonadal differentiation (Czerwinski et al., 2016). In addition, genetic engineering (e.g., RNA interference) has been applied to identify the function of sex-related genes in sex determination in reptiles (Ge et al., 2018; Weber et al., 2020). However, despite several decades of research on the potential underlying mechanisms of TSD in reptiles, the role and function of key sex-related genes in the female sex determination process remain unverified.

Cyp19a1 modulates estrogen to promote sexual differentiation and is both necessary and sufficient for ovarian differentiation in GSD species (Bao et al., 2017). Given its vital role in GSD systems, *Cyp19a1* may be involved in female TSD. Based on previous transcriptomic analyses, *Cyp19a1* is reported to show female-specific expression patterns in early embryonic gonads during late TSP (embryonic developmental stage 19) in TSD turtles (Czerwinski et al., 2016). In contrast, however, *Cyp19a1* transcripts have been detected in adrenal-kidney-gonad complexes (AKGs) after the sex determination TSP in *Alligator mississippiensis* and several TSD turtle species (Gabriel et al., 2001; Murdock & Wibbels, 2003), thereby repudiating its role in sex determination. Therefore, the role of *Cyp19a1* in TSD remains unresolved in reptiles and may differ among species. Moreover, previous studies have only explored mRNA expression of *Cyp19a1* in embryonic gonads and AKGs, which is inadequate for providing unequivocal conclusions regarding *Cyp19a1* function. Here, we performed multi-level analysis (e.g., gene expression patterns, gene thermo-sensitivity, hormone/genetic manipulation) of embryonic gonads to determine the function of *Cyp19a1* in a TSD turtle species (*M. reevesii*).

To investigate the expression patterns of *Cyp19a1*, we analysed expression levels during different developmental embryonic stages (16 to 21) at the male-producing temperature (MPT, 26 °C) and female-producing temperature (FPT, 32 °C). Results showed *Cyp19a1* expression in FPT

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gonads as early as stage 16, i.e., early TSP and clearly prior to gonadal differentiation. Furthermore, *Cyp19a1* expression increased dramatically with embryonic development. *Cyp19a1* expression in the FPT gonads was tens of times higher than that in the MPT gonads at stage 16 and thousands of times higher at stages 19 and 25. In contrast, MPT gonads exhibited extremely low *Cyp19a1* expression throughout embryogenesis (Figure 1A). These results suggest that *Cyp19a1* is not only involved in sexual differentiation, but is also an early responder for female determination in *M. reevesii*.

Cyp19a1 is reported to show temperature dependence between a temperature range of 26 °C to 32 °C in TSD reptiles (Czerwinski et al., 2016). To date, however, how *Cyp19a1* expression is influenced by temperature is not

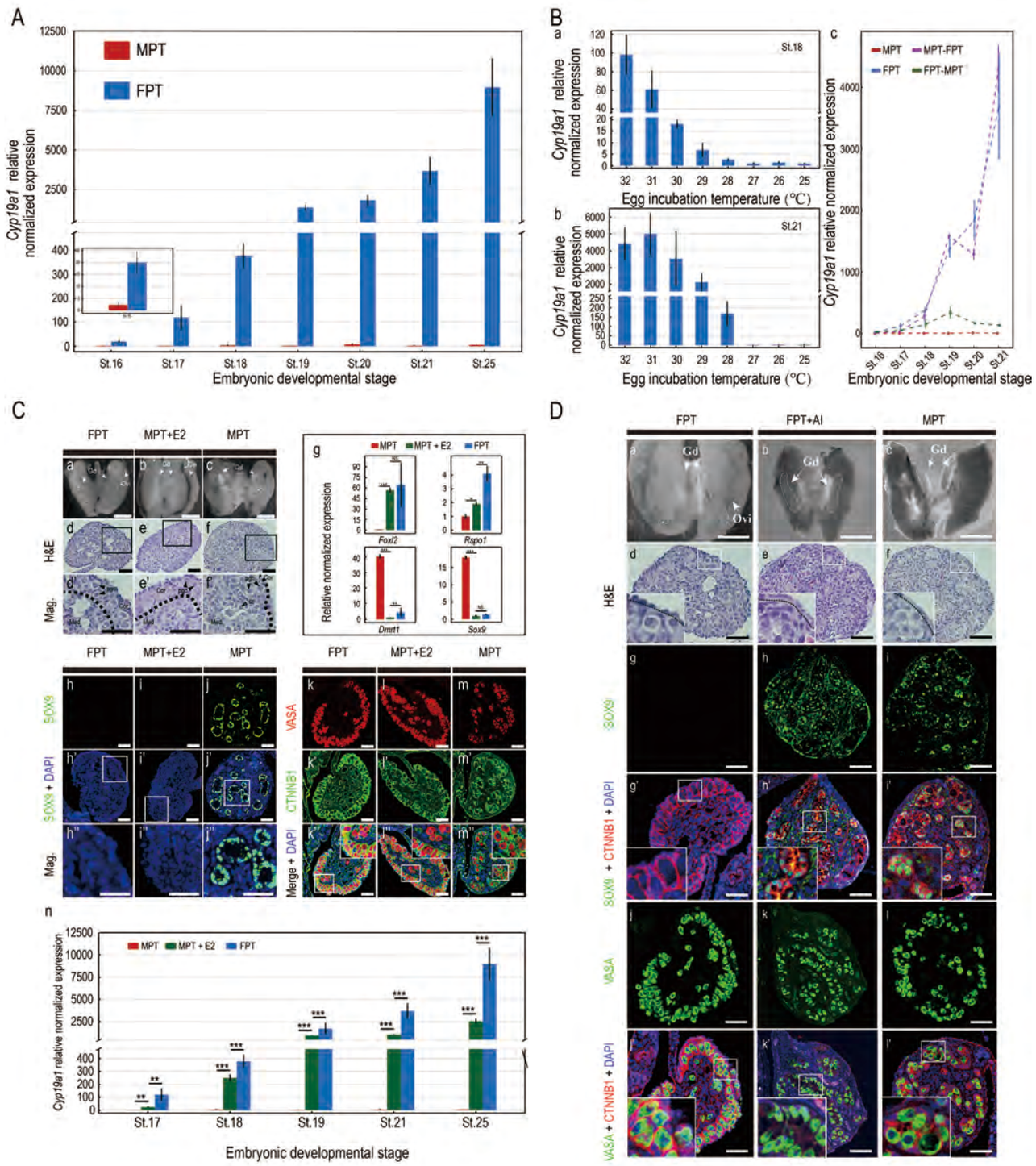

Figure 1 Evidence for the role of *Cyp19a1* in sex determination in *M. reevesii*

A: *Cyp19a1* mRNA expression in gonads at different stages (16–25) at MPT (26 °C) and FPT (32 °C) determined by qRT-PCR. B: Thermo-sensitivity of *Cyp19a1*. a, b: qRT-PCR analysis of *Cyp19a1* in gonads at stages 18 or 21 at gradient temperatures. c: Time-course response of *Cyp19a1* expression to temperature shifts from either MPT→FPT or FPT→MPT. C: Up-regulation of *Cyp19a1* in feminized MPT gonads during male-to-female sex reversal. a–c: Gonads (outlined by white dotted lines) on top of mesonephros of MPT, feminized MPT, and FPT embryos at stage 25. Scale bar: 1 mm. d–f': Hematoxylin and eosin (H&E) staining of gonadal sections from MPT, feminized MPT, and FPT embryos at stage 25. g: qRT-PCR analysis of *Foxl2*, *Rspo1*, *Dmrt1*, and *Sox9* in gonads from MPT, MPT with estrogen treatment, and FPT embryos at stage 25. h–j'': Protein localization of *Sox9* in MPT, feminized MPT, and FPT gonadal sections at stage 25. k–m'': Female-typical distribution of germ cells in MPT gonads with estrogen treatment at stage 25, as determined by *Vasa* and *β-catenin* immunostaining. n: qRT-PCR analysis of *Cyp19a1* in gonads from MPT, feminized MPT, and FPT embryos at stages 17, 18, 19, 21, and 25. D: Masculinization of FPT embryos following aromatase inhibitor treatment. a–c: Representative images of gonad-mesonephros complexes from MPT, FPT with aromatase inhibitor treatment, and FPT embryos at stage 25. Scale bar: 1 mm. d–f: H&E staining of gonadal sections from MPT, FPT with aromatase inhibitor treatment, and FPT embryos at stage 25. g–i': Immunofluorescence of *Sox9* and *β-catenin* in gonadal sections of MPT, FPT with aromatase inhibitor treatment, and FPT embryos at stage 25. j–l′: Immunofluorescence of *Vasa* in MPT, FPT with aromatase inhibitor treatment, and FPT gonads at stage 25. β-actin was used as a reference gene for qRT-PCR analysis. Data are mean±standard deviation (*SD*); n≥3. Dashed line indicates border between medulla and cortex. Gd, gonad; Ovi, oviduct. pgc, primordial germ cells; sc, seminiferous cord; Cor, cortex; Med, medulla. For H&E staining and immunostaining sections, scale bars are 50 μm.

known. In the present study, we showed that *Cyp19a1* expression in turtle gonads decreased obviously with declining temperature (from 31 °C to 27 °C) at both stage 18 (during TSP) and stage 21 (after TSP) (Figure 1Ba, b). In addition, *Cyp19a1* responded rapidly to the temperature shift in the developing gonads when embryos were transferred from MPT to FPT at stage 16. Specifically, *Cyp19a1* increased slightly above the MPT-typical level at early stage 17 (2 days after transfer), then increased significantly at subsequent stages. However, *Cyp19a1* showed a time lag in response to the temperature shift in gonads after FPT-to-MPT transfer, increasing continuously until stage 19 and then decreasing to the MPT-typical level (Figure 1Bc). These findings confirmed the high thermo-sensitivity of *Cyp19a1* in *M. reevesii* gonadal cells, indicating its vital role in the female pathway by connecting temperature and sex in the TSD system.

During the TSP, administration of exogenous estrogen may override the temperature effect to induce feminization of MPT embryos in TSD reptiles (Ramsey & Crews, 2009). Here, we treated MPT embryos of *M. reevesii* with E2 at stage 16 and found that the gonads exhibited female-like morphology, with a degenerated medulla and a thickened outer cortex at stage 25 (Figure 1Ca–f'). A significant up-regulation of *Foxl2* and *Rspo1* and down-regulation of *Dmrt1* and *Sox9* were also detected in feminized MPT embryos at stage 25 (Figure 1Cg). Correspondingly, the protein expression levels of *Sox9* (a Sertoli cell marker) and *Vasa* (a germ cell marker) in the MPT gonads with estrogen treatment exhibited female-like distribution patterns (i.e., no *Sox9* expression, cortical localization of *Vasa*) rather than male patterns (i.e., high expression of *Sox9*, medullary cord distribution of *Vasa*) (Figure 1Ch–m'', Supplementary Figure S3A–F''). Further analysis showed that *Cyp19a1* transcripts responded rapidly to estrogen treatment and were significantly up-regulated from stage 17 (2 days after treatment) onwards (Figure 1Cn). Therefore, it is possible that the reversal of *Cyp19a1* expression by exogenous estrogen may be responsible for sex-reversal in *M. reevesii*. These results suggest that *Cyp19a1* is required for early ovarian determination in TSD.

In GSD species, aromatase inhibitor or *Cyp19a1* loss-of-function treatments can induce permanent female-male sex reversal (Bao et al., 2017; Vaillant et al., 2001). In *M. reevesii*, when FPT embryos were treated with aromatase inhibitor, gonads became shortened and vascularized and exhibited male-like morphology, including dense medulla with several primordial germ cells and degenerated cortex (Figure 1Da–f). The *Sox9* protein was specifically expressed in the nuclei of precursor Sertoli cells in MPT gonads, consistent with the FPT gonads treated by aromatase inhibitor (Figure 1Dg–i'', Supplementary Figure S4A–C''). The distribution of *Vasa*-positive germ cells in FPT gonads following aromatase inhibitor treatment displayed a male-like medulla localization pattern (Figure 1Dj–l'', Supplementary Figure S4D–F''). These findings strongly suggest that *Cyp19a1* is necessary for the female determination process in the TSD turtle species *M. reevesii*.

In vertebrates like fish, amphibians, and birds, *Cyp19a1* generally exhibits a sexually dimorphic expression pattern in female gonads during early embryonic stages and is robustly expressed within the cytoplasm of the ovarian medulla (Jin et al., 2020; Mawaribuchi et al., 2014; Nakamoto et al., 2018). In *Mus musculus*, although *Cyp19a1* is not required for sex determination or gonadal differentiation during the embryonic stage, it is essential for maintenance of granulosa cells and ovarian phenotype in postnatal ovaries (Erickso et al., 1979). In this study, we demonstrated that *Cyp19a1* is a key female sex-determining gene in *M. reevesii* turtles. Therefore, the function of *Cyp19a1* is highly conserved in the sexual differentiation progress, even in a wider phylogenetic context. Our study provides unequivocal evidence for the critical function of *Cyp19a1* in the female pathway in turtles and its importance in female sex determination, thereby shedding light on the elusive molecular mechanism underlying TSD.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

P.F.W., X.F.W., and W.G.D. conceived and designed the study; P.F.W. and X.F.W. performed the experiments; P.F.W. and X.F.W. analyzed the data; and P.F.W., X.F.W., F.G., and W.G.D. cowrote the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

Bao HS, Cai H, Han W, Zhang HY, Sun W, Ge CT, et al. 2017. Functional characterization of *Cyp19a1* in female sexual differentiation in *Pelodiscus sinensis*. *Scientia Sinica Vitae*, **47**(6): 640–649. (in Chinese)

Czerwinski M, Natarajan A, Barske L, Looger LL, Capel B. 2016. A timecourse analysis of systemic and gonadal effects of temperature on sexual development of the red-eared slider turtle *Trachemys scripta elegans*. *Developmental Biology*, **420**(1): 166–177.

Erickso GF, Hsueh AJW, Quigley ME, Rebar RW, Yen SSC. 1979. Functional studies of aromatase activity in human granulosa cells from normal and polycystic ovaries. *The Journal of Clinical Endocrinology & Metabolism*, **49**(4): 514−519.

Gabriel WN, Blumberg B, Sutton S, Place AR, Lance VA. 2001. Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. *Journal of Experimental Zoology*, **290**(5): 439−448.

Ge CT, Ye J, Weber C, Sun W, Zhang HY, Zhou YJ, et al. 2018. The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. *Science*, **360**(6389): 645−648.

Jin K, Zuo QS, Song JZ, Zhang YN, Chen GH, Li BC. 2020. *Cyp19a1* (aromatase) dominates female gonadal differentiation in chicken (*Gallus gallus*) embryos sexual differentiation. *Bioscience Reports*, **40**(10): BSR20201576.

Mawaribuchi S, Ikeda N, Fujitani K, Ito Y, Onuma Y, Komiya T, et al 2014. Cell-mass structures expressing the Aromatase gene *Cyp19a1* lead to ovarian cavities in *Xenopus laevis*. *Endocrinology*, **155**(10): 3996–4005.

Murdock C, Wibbels T. 2003. Cloning and expression of aromatase in a turtle with temperature-dependent sex determination. *General and Comparative Endocrinology*, **130**(2): 109−119.

Nakamoto M, Shibata Y, Ohno K, Usami T, Kamei Y, Taniguchi Y, et al. 2018. Ovarian aromatase loss-of-function mutant medaka undergo ovary degeneration and partial female-to-male sex reversal after puberty. *Molecular and Cellular Endocrinology*, **460**: 104−122.

Pieau C, Dorizzi M, Richard-Mercier N. 1999. Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cellular and Molecular Life Sciences*, **55**(6–7): 887–900.

Ramsey M, Crews D. 2009. Steroid signaling and temperature-dependent sex determination - Reviewing the evidence for early action of estrogen during ovarian determination in turtles. *Seminars in Cell & Developmental Biology*, **20**(3): 283−292.

Shoemaker C, Ramsey M, Queen J, Crews D. 2007. Expression of *Sox9, Mis*, and *Dmrt1* in the gonad of a species with temperature-dependent sex determination. *Developmental Dynamics*, **236**(4): 1055−1063.

Vaillant S, Dorizzi M, Pieau C, Richard-Mercier N. 2001. Sex reversal and aromatase in chicken. *Journal of Experimental Zoology*, **290**(7): 727−740.

Weber C, Zhou YJ, Lee JG, Looger LL, Qian GY, Ge CT, et al. 2020. Temperature-dependent sex determination is mediated by pSTAT3 repression of *Kdm6b*. *Science*, **368**(6488): 303–306.