Original Article

Effects of Incubation Temperature on the Expression of Sex-Related Genes in the Chinese Pond Turtle, *Mauremys reevesii*

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Abstract

Despite widespread temperature-dependent sex determination (TSD) in reptiles, it is still unclear how the molecular network responds to temperature variation and drives the sexual fate. Profiling of sex-related genes is the first step in understanding the sex determination system in reptiles. In this study, we cloned the full-length coding sequences of *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* in an Asian freshwater turtle (*Mauremys reevesii*) with TSD and identified the expression patterns of these genes and *Dmrt1* at different incubation temperatures to understand their roles in urogenital development. Our results showed that *Cyp19a1*, *Foxl2,* and *Rspo1* were expressed in the adrenal-kidney-gonadal complex at a high level in females, while *Sf1* and *Dmrt1* were highly expressed in males. In addition, *Foxl2* and *Rspo1* showed sex-dimorphic expression in the presumed early thermosensitive period (TSP), *Dmrt1* was upregulated at the beginning of the presumed TSP, and *Sox9* did not show sex-dimorphic expression until the end of the presumed TSP. These results suggest that *Foxl2* and *Rspo1* are probably upstream genes involved in female sex determination and that *Dmrt1* may be a key factor in male sex determination. Therefore, our study provides a solid foundation for further investigations on the molecular mechanism underlying sex determination in *M. reevesii*.

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Vertebrate mechanisms of sex determination range between 2 main extremes: genotypic sex determination (GSD) and environment-dependent sex determination (ESD) [Bull, 1980; Wilhelm et al., 2007]. Mammals and birds exhibit GSD, but they have quite different key factors that control their sexual fate. The sex of a therian mammalian embryo is determined by the existence of the *Sry* gene located on the Y chromosome, with heteromorphic sex chromosomes (XY) determining an individual to be male [Kashimada and Koopman, 2010]. In birds, no homolog of *Sry* has been found. Dosage-dependent *DMRT1* on the Z chromosome is considered to determine the male fate, and heteromorphic sex chromosomes (ZW) determine a female [Smith and Sinclair, 2001]. In medaka fish, *dmy* (the DM-domain gene on Y chromosome) is required for male sex determination [Matsuda et al., 2002]. In contrast, GSD and ESD coexist in different

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lineages of reptiles, with GSD prevailing in squamates and ESD in all crocodiles, most turtles, and some lizards [Ferguson and Joanen, 1982; Janzen and Paukstis, 1991; Viets et al., 1993]. ESD mainly occurs as temperature-dependent sex determination (TSD) in reptiles, in which the sex of the offspring is determined by the incubation temperature in the thermosensitive period (TSP) [Viets et al., 1993].

The sex-determining system has been investigated in a number of reptiles, from turtles to crocodilians [Valenzuela, 2004]. These studies identified 3 models of TSD: (1) TSDIa – MF type, with males at low incubation temperatures and females at high temperatures [Willingham et al., 2000; Du et al., 2007, 2010]; (2) TSDIb – FM type, the opposite of TSDIa [Mitchell et al., 2006]; and (3) TSDII – FMF type, with females at both low and high temperatures and males at intermediate temperatures [Ferguson and Joanen, 1982]. Despite widespread occurrence of TSD in reptiles, it is still unclear how the molecular network responds to temperature and drives the sexual fate. Our understanding of the molecular network in reptiles is mostly based on previous studies that investigated sex determination in mammals, although recent transcriptomic approaches have examined the alligator (*Alligator mississippiensis*) [Yatsu et al., 2016], slider turtle (*Trachemys scripta*) [Czerwinski et al., 2016], and painted turtle (*Chrysemys picta*) [Radhakrishnan et al., 2017] genomes directly. Earlier studies suggested that in therian mammals the high expression of S*ry* upregulates the expression of *Sox9* and downstream genes such as *Dmrt1* [Bullejos and Koopman, 2001]. Then, *Sox9* interacts with *Sf1* to upregulate the expression of *Amh* (also known as *Mis*), which controls the regression of the female ductal primordium [Sekido and Lovell-Badge, 2008]. Previous studies have evaluated sex-related genes in different lineages of turtles [Bull et al., 1988; Valenzuela et al., 2013], lizards [Rhen and Crews, 2001; Endo et al., 2008], and in crocodiles [Western et al., 1999a; Urushitani et al., 2011] that undergo TSD by using a candidate gene approach. Although *Sry* has no homologs outside therian mammals, more than 15 homologous genes involved in the sex determination pathway of mammals were identified in reptiles [Capel and Tanaka, 2013]. Amongst these sex-related genes, *Sox9, Dmrt1,* and *Amh* are expressed at a high level in males, and *Foxl2, Cyp19a1,* and *Rspo1* are highly expressed in females [Crews et al., 2001; Shoemaker et al., 2007a; Smith et al., 2008; Valenzuela et al., 2013].

Research on the molecular networks in sex determination in reptiles (especially TSD) is still in the early stages.

Importantly, from the existing data it is evident that genes involved in sex determination are expressed differently not only among distant vertebrate lineages but also among closely related species [Valenzuela et al., 2013]. For example, *Sox9* expression precedes *Amh* expression in mammals, but the opposite trend can be observed in birds and alligators [Western et al., 1999a]. Evidence shows that, even in related species, the key genes working as the sex determination trigger can be very different. For example, 2 closely related species of medaka have different key sex-determining genes, namely *dmy* in *Oryzias latipes* and *gsdf* in *O. luzonensis* [Myosho et al., 2012]. Accordingly, profiling of homologous sex-related genes in different species is important for understanding the sex determination system and its evolution in vertebrates. Yet, a vast majority of the studies of gene expression in TSD reptiles concentrated on few species, mostly turtles of the family Emydidae (e.g., *T. scripta, C. picta*), plus a few other turtles, crocodilians (e.g., *A. mississippiensis*) and lizards (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000479360). Most of these turtles are found in North America. For example, transcriptome analysis revealed that genes involved in steroid enzyme synthesis and brain development were sexually dimorphic expressed prior to TSP in *T. scripta* [Czerwinski et al., 2016], similar to what is observed for *Sf1*, *Wt1*, and *Dax1* involved in gonadal development in *C. picta* [Valenzuela et al., 2006; Valenzuela, 2008a, b], and it also revealed that *Cirbp* (cold-inducible RNA-binding protein) is involved in sex determination in *C. serpentina* [Schroeder et al., 2016]. But there are few studies on the molecular networks of TSD reptiles in other regions such as Asia [Hoshi and Nakao, 2008; Inamdar et al., 2015]. Thus, molecular studies across TSD reptiles representing other turtle families, reptilian lineages, and geographic regions are overdue and necessary if we are to fully understand the evolution of TSD.

As a step to help fill this gap, here we studied the sex-related genes of the Chinese three-keeled pond turtle, *Mauremys reevesii* (TSDIa – MF type). To our knowledge, this is the first such study in a chelonian of the family Geomydidae. Specifically, we cloned full open reading frames (ORFs) of sex-related genes such as *Cyp19a1* (*aromatase*), *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* and investigated the expression patterns of these genes as well as of the previously characterized *Dmrt1* [Hoshi and Nakao, 2008] during the TSP at different incubation temperatures to assess the potential roles of these genes in the sex determination of *M. reevesii*. These genes were chosen because they play critical roles in sex determination in other vertebrates, including TSD

reptiles. For instance, *Rspo1* and *Sox9* sit on opposite sides of the balance in early vertebrate sex determination that is then tipped towards the male or female pathway [DiNapoli and Capel, 2008]. *Sf1* is key for the formation of the bipotential gonad and displays one of the earliest thermosensitive expression patterns in TSD turtles [Valenzuela et al., 2006]. *Cyp19a1* is indispensable for ovarian formation, and its activity is thermosensitive in TSD reptiles [Desvages and Pieau, 1992; Smith et al., 1995], and *Foxl2* and *Dmrt1* are candidate transducers of the environmental temperature into TSD and are critical for female and male development, respectively [Matsumoto and Crews, 2012]. Profiling of these genes and their potential functions in the sex-determining pathways provides a solid basis for further investigations on the molecular network underlying TSD in reptiles.

Materials and Methods

Egg Collection and Incubation
In May 2014, we obtained 120 freshly laid eggs from mixed 15–30 clutches of the three-keeled pond turtle (*M. reevesii*) from a private turtle farm in Hangzhou, Zhejiang province. The eggs were maintained in vermiculite and transported to the laboratory. All eggs were candled, and viable eggs with a white fertilization spot on top of the egg shell were randomly placed in trays (220 × 100 × 80 mm) containing 1:1 vermiculite and water. The trays were then transferred to 2 incubators (KB240; Binder, Germany) set at 26°C (male-producing temperature [MPT]) and 30°C (female-producing temperature [FPT]) [Du et al., 2007]. Additional eggs (5–10) laid the same day were opened to assess their developmental stage, and all embryos were at a stage earlier than stage 12 [Greenbaum, 2002] at the onset of the incubation experiments. The temperatures of the incubators were monitored daily with thermal data loggers (iButton Thermochron, DS 1921; MAXIM Integrated Products Ltd.). The egg trays were rotated twice a week within the incubator to avoid small temperature-gradient effects.

Tissue Collection
Embryonic development was monitored by periodic dissection of randomly selected eggs every other day after 1 week of incubation. The developmental stages of the embryos were determined according to the staging criteria previously established for *T. scripta* [Greenbaum, 2002]. A total number of 108 embryos were harvested across stages 15, 16, 17, 19, 21, and 23 (9 embryos per stage per temperature), and the adrenal-kidney-gonad (AKG) complex of each embryo was rapidly dissected and immediately frozen in liquid nitrogen. The AKG complex has been utilized as a tissue source to investigate the expression of genes involved in sexual development [Murdock and Wibbels, 2006]. While using the AKG complex can mask differential expression from the gonad alone when expression is present in the larger adrenal-kidney portion [Valenzuela et al., 2013], valuable insights can be gained from differential expression in AKG complex data that help develop a working hypothesis for future research. Here, we used the AKG complex as an initial step

Table 1. Degenerate primer sequences used to clone the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′
Aromatase-dg-sense	GGGCTCAGTTACAGGACCT
Aromatase-dg-antisense	TGTCCAGATGGTCTTTCG
Foxl2-dg-sense	GAAGCCGGACCCSKCKCAG
Foxl2-dg-antisense	GAAGCCRGACTGCAGGTAC
Rspo1-dg-sense	TAGCCTGGGCAATCTTTACGAG
Rspo1-dg-antisense	CCATTGTAAAGTCCTTGGCATT
Sf1-dg-sense	GGACCGTGCAGAATAACAAG
Sf1-dg-antisense	TGTGTTAAGCCAAATCGATGC
Sox9-dg-sense	GAAGCAAGACCTGAAGCGAGA
Sox9-dg-antisense	ACGAGTTGGCCGGTGAGA

Table 2. RACE primer sequences used to clone the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′
Aromatase-3race-1-senses	CCTTTGGATGGGAGTGGG
Aromatase-3race-2-senses	GTCCTGGTCTCGTGCGTATG
Foxl2-5race-1-senses	CCTTCTCGAACATGTCCTCGCAG
Foxl2-5race-2-senses	GGGCACCTTGATGAAGCACTCG
Foxl2-3race-1-senses	GGCAGAACAGCATCCGCCACAACC
Foxl2-3race-2-senses	ACGGCTACCTCTCCCCGCCCAAG
Sf1-5race-1-senses	GACCTCAGGGATGTCGGGTGGGG
Sf1-5race-2-senses	TGGATGCCGTGGATGGTGGAGGAC
Sox9-5race-1-senses	GCTCAGGGTGGTCATCGTG
Sox9-5race-2-senses	GCCGCTGTAGGTGACTTGG

to explore the expression of sex-related genes in *M. reevesii*. While the presence of TSD in *M. reevesii* is well documented [Du et al., 2007], no study has determined the TSP for this turtle. Thus, we assumed that the target stages sampled here may encompass the early, middle, and late TSP in this species as it occurs in other cryptodiran turtles [Bull and Vogt, 1981], but we note that because the TSP can vary among closely related species [Bull and Vogt, 1981; Bieser and Wibbels, 2014; Gomez-Saldarriaga et al., 2016], further testing is needed to corroborate the validity of this assumption.

Total RNA Extraction and cDNA Synthesis
Three AKG complexes were pooled together as a sample and homogenized in a mortar with liquid nitrogen. Three of these pooled samples were extracted for each developmental stage at different incubation temperatures. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's

Table 3. Real-time PCR primer sequences for the genes related to sex determination in *Mauremys reevesii*

	Sequence 5′–3′	Product length, bp[a]
β-actin-RT-sense β-actin-RT-antisense	CTGCTCACTGAAGCCCCCC GGCGTAGCCCTCGTAGATTGG	201
Aromatase-RT-sense Aromatase-RT-antisense	TCCTTCTCGCTACTTTCAGCCATTTG ATAAGGGCTGTCTTTCATTCGGGTG	197
Foxl2-RT-sense Foxl2-RT-antisense	CCGGCATCTACCAGTACATCATCAGC GGGCACCTTGATGAAGCACTCGTT	116
Rspo1-RT-sense Rspo1-RT-antisense	TCCACCTGCCCAGAAGGCTACAC TCGTTGCCTTTCTTGAAGCCACAC	140
Sf1-RT-sense Sf1-RT-antisense	TGTCCTCCACCATCCACGGC GGGAAAGGAGGGGTAATGGTAGC	167
Dmrt1-RT-sense Dmrt1-RT-antisense	TGGAAAGCAACAGCCCAACAC ATGGCTGGTAAAAACTGCCGTAG	174
Sox9-RT-sense Sox9-RT-antisense	GACCATCACCCGCTCGCAG GCGTGTACATGGGCCTTTGG	137

[a] According to Udar et al. [2003].

protocol. The quality and quantity of each total RNA sample were assessed using a spectrophotometer (Thermo) at 260 and 280 nm. Then, the total RNA was treated with gDNA eraser to eliminate genomic DNA contamination, and single-stranded cDNA was reverse-transcribed using the RT reagent kit with gDNA eraser (Ta-KaRa Bio Inc.) according to the manufacturer's protocol.

Cloning of Turtle Gene Homologs

Degenerate primers for *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, and *Sox9* were designed on conserved regions based on the sequences of *T. scripta*, *C. picta*, *A. mississippiensis*, *Lepidochelys olivacea*, and human (Table 1). Primers were used to amplify cDNA fragments of 646 bp of *Cyp19a1* (annealing: 52°C, extension: 0.5 min), 415 bp of *Foxl2* (annealing: 58°C, extension: 0.5 min), 1,600 bp of *Sf1* (annealing: 56°C, extension: 1.5 min), 974 bp of *Sox9* (annealing: 59°C, extension: 1 min), and the full length of *Rspo1* (1,632 bp; annealing: 59°C, extension: 1.5 min). The PCR cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 52–59°C for 30 s, 72°C for 0.5–1.5 min, and then 72°C for 7 min. Then, 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed to obtain the full length of *Cyp19a1*, *Foxl2*, *Sf1*, and *Sox9* by using the SMART RACE Kit (Clontech, TaKaRa Bio) according to the manufacturer's instructions. The primers used for RACE were designed on the basis of the fragments of each gene (Table 2). The amplified fragments were separated using gel electrophoresis and then purified with the QuickGel Extraction Kit (CWBIO). The purified fragments were cloned into the pEASY-T1 vector (Transgen) and sequenced using M13F and M13R primers. For *Dmrt1*, we used the full length sequence (GenBank: AB365876.1) previously cloned by others [Hoshi and Nakao, 2008].

Quantitative Real-Time PCR

AKG complexes from 3 individual turtles were pooled into one sample, and 3 such samples were used to quantify gene expression

via QRT-PCR per stage for each temperature treatment. Relative gene expression levels were assessed using SYBR Green (Bio-Rad) according to the manufacturer's protocol and the Roche LightCycler 480 real-time PCR cycler (Roche). All samples were amplified in triplicate, and the expression data from the target genes were normalized to the constitutive expression of β-actin. The relative gene expression levels were calculated using the equation $2^{-\triangle\triangle CT}$. The primers (Table 3) used to assay gene expression were designed using Primer Premier 5.0 (PremierBiosoft). The quantitative PCR cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 63°C for 20 s, and then a final melting curve from 65–95°C to assess that single products were amplified by the qPCR reactions. Specificity was verified further using agarose gel electrophoresis.

Statistical Analysis

We conducted factorial analysis of variance to detect the influence of temperature and developmental stage on the expression of the sex-related genes. The data are presented as means ± one standard error, and statistical significance was assessed at α = 0.05.

Results

Isolation of cDNA

After performing RT-PCR and RACE, we obtained the full lengths of the 5 genes (available in NCBI GenBank). Overall, nucleotide and amino acid sequences from *M. reevesii* closely resemble those reported for other turtles and were increasingly disparate to those of alligator, chicken, mouse, and human as described below.

Tang/Mu/Valenzuela/Du

Fig. 1. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human CYP19A1. The box demarcates the conserved domain of the cytochrome P450 superfamily. Accession numbers for the sequences used are turtle (*Trachemys scripta*), AAG09376; alligator (*Alligator mississippiensis*), NP001274195; chicken (*Gallus gallus*), NP001001761; mouse (*Mus musculus*), NP031836; human (*Homo sapiens*), NP000064.

Cyp19a1

A total of 1,995 bp of nucleotide sequence (GenBank: KU821113) and 504 of deduced amino acid sequence were obtained (online suppl. Fig. 1). The deduced amino acid sequence was 97, 86, 82, 73, and 76% identical to *T. scripta*, alligator, chicken, mouse, and human aromatase, respectively (Fig. 1). The conserved domain of the cytochrome P450 superfamily was identical between *M. reevesii* and *T. scripta*.

Foxl2

A total of 2,224 bp of nucleotide sequence (GenBank: KU821114) and 301 of deduced amino acid sequence were obtained (online suppl. Fig. 2). The deduced amino acid sequence was 99, 84, 86, 70, and 66% identical to *C. picta*, alligator, chicken, mouse, and human FOXL2, re-

spectively (Fig. 2). The forkhead domain was highly conserved across vertebrates, with only a single amino acid change at position 86 in mammals.

Rspo1

A total of 1,633 bp of nucleotide sequence (GenBank: KU821115) and 260 of deduced amino acid sequence were obtained (online suppl. Fig. 3). The deduced amino acid sequence was 98, 85, 87, 65, and 68% identical to *C. picta*, alligator, chicken, mouse, and human RSPO1, respectively (Fig. 3). The conserved domain of Furin-like repeats was identical between *M. reevesii* and *C. picta*.

Sf1

A total of 1,812 bp of nucleotide sequence (GenBank: KU821116) and 466 of deduced amino acid sequence

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Mauremys    MMASYPDPEE DPVALMAHDT NAS-KEAERA KDELSPEKG- -----AEKPD PSQKPPYSYV ALIAMAIRES AEKRLTLSGI YQYIISKFPF YEKNKKGWQN [100]
Chrysemys   .....Q.... .......... ...-...... .........- ----....... ...... ........... .......... .......... .......... [100]
Alligator   -..G..E.D. A.A..L..G GGGG...... .E.PGAD..- -----.D... ...... .......... .......... .......... .......... [100]
Gallus      ..SG.A.G.. .A..ML...G GG.-..P..G .E...A..- -----P.... ...... .......... .......... .......... .......... [100]
Mus         ......E..D TAGT.L.PES GRAV....AS PPSPGKGG.- ---TTP.... .A. ...... .......... .......... A......... .......... [100]
Homo        ......E..D AAG...L.PE. GRTV..P.GP PPSPGKGG.G GGGTAP.... .A. ...... .......... .......... A......... .......... [100]

Mauremys    SIRHNLSLNE CFIKVPREGG GERKGNYWTL DPACEDMFEK GNYRRRRMK RPFRPPPTHF QPGKTLFSS- ---------- ---DSYGYLS PPKYLQSGFM [200]
Chrysemys   .......... .......... .......... .......... .........- ........... .........- ---------- ---....... .......... [200]
Alligator   .......... .......... .......... .......... .........A. ....G..GG- ---------- ---.G..... .......... [200]
Gallus      .......... .......... .......... .......... .........S..GP- ---------- ---.G..... .......T.. [200]
Mus         .......... .......... .......... .......... .........A. ....G..G.G GAAGGCGVPG AGA.G....A .........L [200]
Homo        .......... .......... .......... .......... .........A. ....G..GAG GAAGGCGVAG AGA.G....A .........L [200]

Mauremys    NNSWPLAQPP APMSYASCQL AGGNVSPVN- ---------- ----VKGLSA P-ASYGPYSR VQGMALP-GM VNSYNGMSH- ---------- -------HHH [300]
Chrysemys   .......... .......... .........- ---------- ----...... .-......... .......-.. .........- ---------- -------..- [300]
Alligator   ....G..... ..VP.....M S..S.....- ---------- ---.....G .-........ ....-.V ......VG.P ---------- ---HHP.A. [300]
Gallus      ......P... ..VP.....M S..S.....- ---------- ---.....G .-........ ....SV..-. ......VA.P ---------- ----HHP.A. [300]
Mus         ......P... S..P.....M .AAAAAAAA AAAAGPGSPG AAAV....AG .A........ ....S....P.V ......LGGP PAAPPPPPPP PHPHPHP.A. [300]
Homo        ......P... S..P.....M .AAAAAAAA AAAAGPGSPG AAAV....AG .A........T. .S....P.V ......LGGP PAAPPPP--- PHPHPHP.A. [300]

Mauremys    HP-------- ---------- --QQLSPASP APLAAPAPN- --GAGLQFTC ARQPAELSMM HCSYWDHDSK HSALHSRIDI [380]
Chrysemys   ..-------- ---------- --....P.... ...-........- --.....A. .......... .......... .......... [380]
Alligator   ..HA------ ---------- --...G.... .P..P..A.- --.....A. .......... ....E.... .G....... [380]
Gallus      .-------- ---------- --...G.... .P....A.- --.....A. .......... ...V...E.... .G....... [380]
Mus         .LHAAAAPPP APPHHGAAAP PPG....... .TA.P...AP TSAP....A. ....-..A. .......... TG.....L.L [380]
Homo        .LHAAAAPPP APPHHGAAAP PPG....... .TA.P...AP TSAP....A. ....-..A. .......... TG.....L.L [380]
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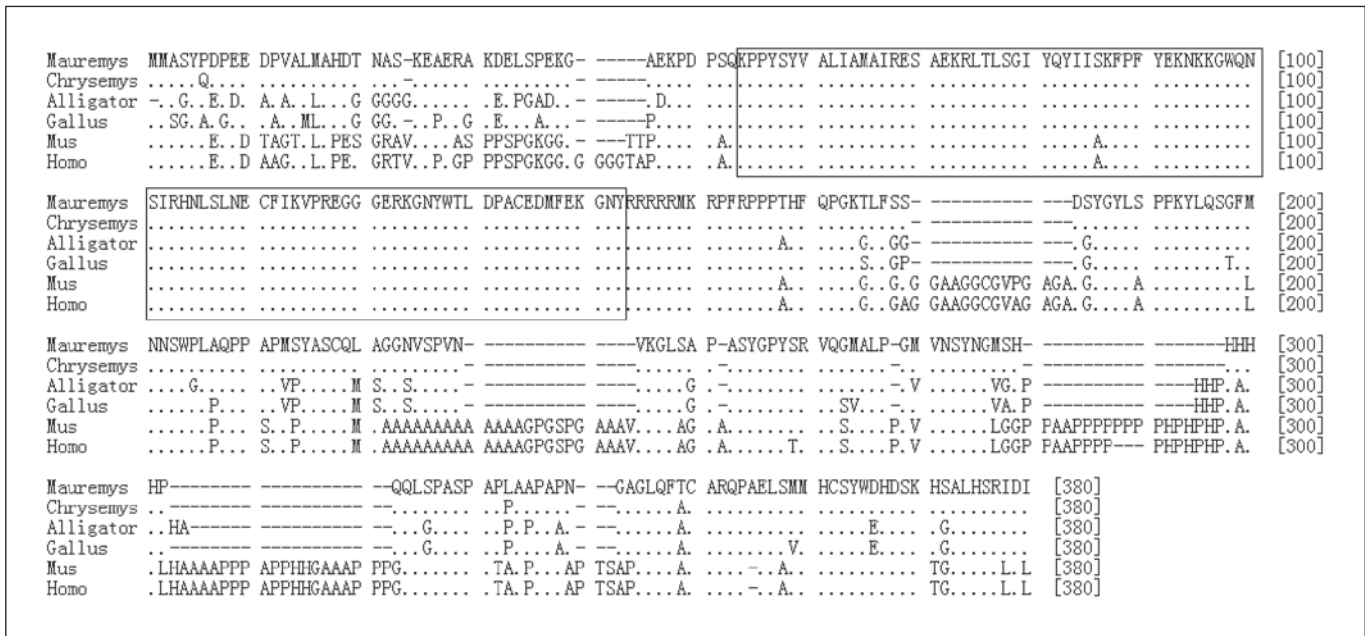
Fig. 2. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human FOXL2. The box demarcates the forkhead domain. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005282573; alligator (*Alligator mississippiensis*), XP006258874; chicken (*Gallus gallus*), NP001012630; mouse (*Mus musculus*), NP036150; human (*Homo sapiens*), NP075555.
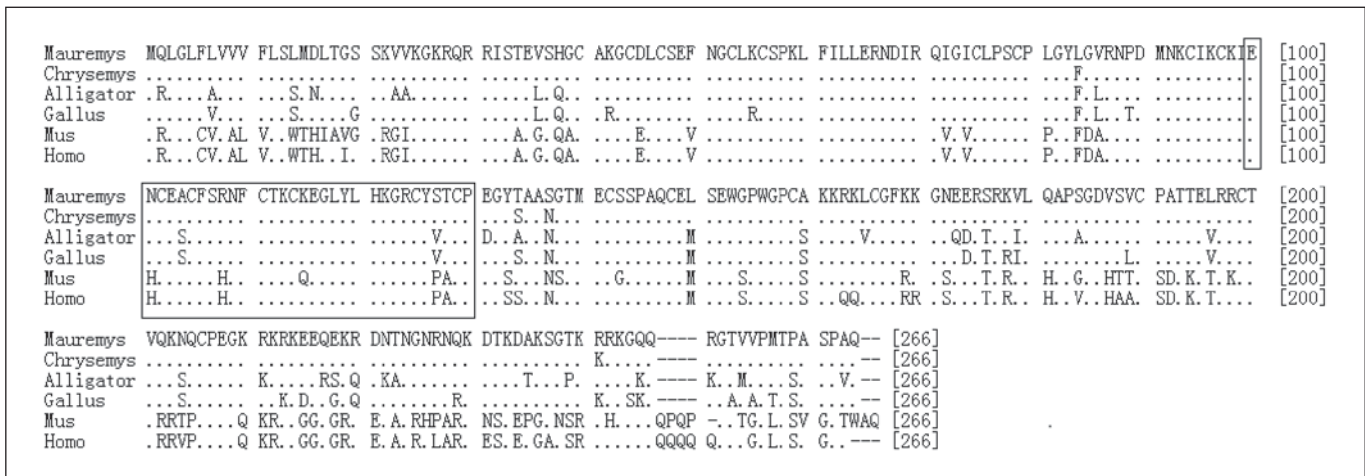
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Mauremys    MQLGLFLVVV FLSLMDLTGS SKVVKGKRQR RISTEVSHGC AKGCDLCSEF NGCLKCSPKL FILLERNDIR QIGICLPSCP LGYLGVRNPD MNKCIKCKIE [100]
Chrysemys   .......... .......... .......... .......... .......... .......... .......... .......... ...F..... ......... [100]
Alligator   .R...A... ...S.N.... ...AA...... .........L.Q.. .......... .......... .......... ...F.L.... .......... [100]
Gallus      .....V.... ...S.....G .......... ........L.Q...R. .........R. .......... .......... ...F.L..T. .......... [100]
Mus         .R...CV.AL V..WTHIAVG .RGI...... ..A.G.QA. ....E....V .......... .......... .V.V...... P..FDA.... ......... [100]
Homo        .R...CV.AL V..WTH..I. .RGI...... ..A.G.QA. ....E....V .......... .......... .V.V...... P..FDA.... ......... [100]

Mauremys    NCEACFSRNF CTKCKEGLYL HKGRCYSTCP EGYTAASGTM ECSSPAQCEL SEWGPWGPCA KKRKLCGFKK GNEERSRKVL QAPSGDVSVC PATTELRRCT [200]
Chrysemys   .......... .......... .......... ..S..N.... .......... .......... .......... .......... .......... .......... [200]
Alligator   ...S...... .......... ......V... D..A..N.... .......M .......... S ....V...... QD.T..I. ...A....... ......V... [200]
Gallus      ...S...... .......... ......V... ..S..N.... .......M .......... S .......... D.T.RI. .......L. ......V... [200]
Mus         H.....H.. ....Q..... ......PA.. ..S...NS.. ...G.....M ...S.....S .........R. .S...T.R. H..G..HTT. SD.K.T.K.. [200]
Homo        H.....H.. .......... ......PA.. ..SS..N... .......M ...S.....S ...QQ....RR .S...T.R. H..V..HAA. SD.K.T.... [200]

Mauremys    VQKNQCPEGK RKRKEEQEKR DNTNGNRNQK DTKDAKSGTK RRKGQQ---- RGTVVPMTPA SPAQ-- [266]
Chrysemys   .......... .......... .......... .......... K..... ---- .......... ....-- [266]
Alligator   ...S...... K.....RS.Q .KA....... ....T...P. ...K.---- K..M....S. ..V.-- [266]
Gallus      ...S...... ....K.D..G.Q .........R. .......... K..SK. ---- ..A.A.T.S. ....-- [266]
Mus         .RRTP....Q KR..GG.GR. E.A.RHPAR. NS.EPG.NSR .H....QPQP -..TG.L.SV G.TWAQ [266]
Homo        .RRVP....Q KR..GG.GR. E.A.R.LAR. ES.E.GA.SR ......QQQQ Q...G.L.S. G..--- [266]
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Fig. 3. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human RSPO1. The box demarcates the conserved domain of Furin-like repeats. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005298408; alligator (*Alligator mississippiensis*), XP006264933; chicken (*Gallus gallus*), NP001305373; mouse (*Mus musculus*), NP619624; human (*Homo sapiens*), NP001033722.

were obtained (online suppl. Fig. 4). The deduced amino acid sequence was 98, 93, 88, 70, and 71% identical to *C. picta*, alligator, chicken, mouse, and human SF1, respectively (Fig. 4). The DNA-binding domain was identical between *M. reevesii* and *C. picta*; only 2 amino acid changes were identified in alligator and mammals, while the ligand-binding domain was less conserved.
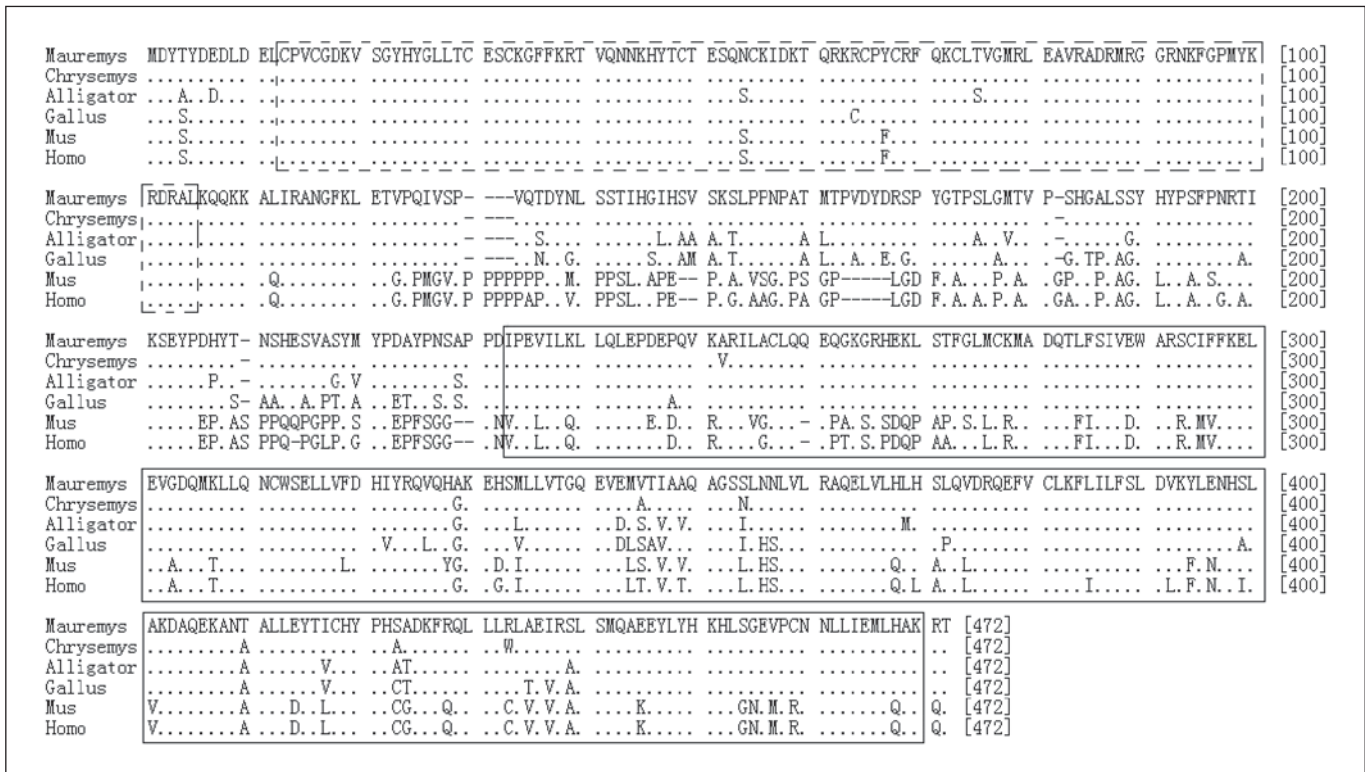
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Mauremys   MDYTYDEDLD ELCPVCGDKV SGYHYGLLTC ESCKGFFKRT VQNNKHYTCT ESQNCKIDKT QRKRCPYCRF QKCLTVGMRL EAVRADRMRG GRNKFGPMYK [100]
Chrysemys  .......... ...|...... .......... .......... .......... .......... .......... .......... .......... .........| [100]
Alligator  ...A..D... ...|...... .......... .......... .......... ......S... .......... ....S..... .......... .........| [100]
Gallus     ...S...... ...|...... .......... .......... .......... .......... .....C.... .......... .......... .........| [100]
Mus        ...S...... ...|...... .......... .......... .......... ......S... .......F.. .......... .......... .........| [100]
Homo       ...S...... ...|_____ _____ _____ _____ _____S___ _____F__ _____ _____ _____| [100]

Mauremys   RDRALKQQKK ALIRANGFKL ETVPQIVSP- ---VQTDYNL SSTIHGIHSV SKSLPFNPAT MTPVDYDRSP YGTPSLGMTV P-SHGALSSY HYPSFPNRTI [200]
Chrysemys  .....|.... .......... ...- --- .......... .......... .......... ....-..... .......... ..........A [200]
Alligator  .....|.... .......... ...- --.S. .....L.AA A.T......A L........ ....A..V.. .-......G. .......... [200]
Gallus     .....|.... .......... ---.N..G. ...S..AM A.T...... A.L..A..E.G. .........A.. .-G.TP.AG. .........A. [200]
Mus        .....|.... .Q........ ...G.PMGV.P PPPPPP..M. PPSL.APE-- P.A.VSG.PS GP-----LGD F.A....P.A. .GP..P.AG. L..A.S.... [200]
Homo       .....|.... .Q........ ...G.PMGV.P PPPPAP..V. PPSL..PE-- P.G.AAG.PA GP-----LGD F.A.A.P.A. .GA..P.AG. L..A..G.A. [200]

Mauremys   KSEYPDHYT- NSHESVASYM YPDAYPNSAP DIPEVILKL LQLEPDEPQV KARILACLQQ EQGKGRHEKL STFGLMCKMA DQTLFSIVEW ARSCIFFKEL [300]
Chrysemys  .........- .......... ........S. .......... .......V.. .......... .......... .......... .......... .......... [300]
Alligator  ......P..- ......G.V ........S. .......... .......A. .......... .......... .......... .......... .......... [300]
Gallus     ........S- AA..A.PT.A ..ET..S.S. .......... .......... A. .......... .......... .......... .......... [300]
Mus        .....EP.AS PPQQPGPP.S ..EPFSGG-- .NV..L..Q. .....E.D. R...VG...- .PA.S.SDQP AP.S.L.R. ...FI...D. ..R.MV.... [300]
Homo       .....EP.AS PPQ-PGLP.G ..EPFSGG-- .NV..L..Q. .....D. R...G...- .PT.S.PDQP AA...L.R. ...FI...D. ..R.MV.... [300]

Mauremys   EVGDQMKLLQ NCWSELLVFD HIYRQVQHAK EHSMLLVTGQ EVEMVTIAAQ AGSSLNNLVL RAQELVLHLH SLQVDRQEFV CLKFLILFSL DVKYLENHSL [400]
Chrysemys  .......... .......... ........G. .......... ...A. ...N. .......... .......... .......... .......... [400]
Alligator  .......... .......... ........G. ...L. ..D.S.V.V. ...I. .........M. .......... .........P. .......... [400]
Gallus     .......... .......... .V..L..G. ...V. ...DLSAV. ...I.HS. .........P. .......... .......... ...........A. [400]
Mus        ..A..T... .........L. .......YG. .D.I. ...LS.V.V. ...L.HS. .......Q. .A..L. ........... ...F.N... [400]
Homo       ..A..T... .......... ...G. .G.I. ...LT.V.T. ...L.HS. ...Q.L.A..L. ....I..... .L.F.N..I. [400]

Mauremys   AKDAQEKANT ALLEYTICHY PHSADKFRQL LLRLAEIRSL SMQAEEYLYH KHLSGEVPCN NLLIEMLHAK RT [472]
Chrysemys  .........A .......... ..A....... ...W...... .......... .......... .......... .. [472]
Alligator  .........A ...V... ..AT...... .......A. .......... .......... .......... .. [472]
Gallus     .........A ...V... ..CT...... ....T.V.A. .......... .......... .......... .. [472]
Mus        V........A ...D..L... ..CG...Q. ..C.V.V.A. ....K..... ...GN.M.R. .......Q. Q. [472]
Homo       V........A ...D..L... ..CG...Q. ..C.V.V.A. ....K..... ...GN.M.R. .......Q. Q. [472]
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Fig. 4. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human SF1. The dashed box demarcates the N-terminal DNA-binding domain and the solid box demarcates the C-terminal ligand-binding domain. Accession numbers for the sequences used are turtle (*Chrysemys picta*), XP005279455; alligator (*Alligator mississippiensis*), XP006271373; chicken (*Gallus gallus*), NP990408; mouse (*Mus musculus*), NP620639; human (*Homo sapiens*), NP004950.

Sox9

A total of 2,053 bp of nucleotide sequence (GenBank: KU821117) and 301 of deduced amino acid sequence were obtained (online suppl. Fig. 5). The deduced amino acid sequence was 98, 95, 90, 85, and 85% identical to *T. scripta*, alligator, chicken, mouse, and human SOX9, respectively (Fig. 5). As expected, the SOX-TCF HMG-box was highly conserved between vertebrates.

Gene Expression Patterns at Different Incubation Temperatures

The expression of several putative genes involved in TSD was analyzed using RT-PCR (Fig. 6) across stages 15–23 (the morphological status of the developing gonad across stages 16–23 is shown in online suppl. Fig. 6), which we presume encompass the TSP in *M. reevesii*. The expression level of *Cyp19a1* was low throughout the presumptive TSP at MPT. However, it was low in the early presumptive TSP and increased after stage 17, reaching its peak at stage 21 at FPT. As a result, the ex-

pression level of *Cyp19a1* was significantly higher at FPT than at MPT ($F_{1, 24} = 44.40$, $p < 0.001$; Fig. 6C). Similar to the expression level of *Cyp19a1*, the level of *Foxl2* was significantly higher at FPT than at MPT ($F_{1, 24} = 37.98$, $p < 0.001$; Fig. 6A). At FPT, the expression level of *Foxl2* increased at stage 16 and remained high until the end of the presumptive TSP. *Rspo1* was another gene that showed high-level expression in females ($F_{1, 24} = 50.47$, $p < 0.001$; Fig. 6B), with its expression reaching a peak at stage 19 of FPT but remaining low at MPT. In contrast, the expression level of *Sf1* was higher at MPT than at FPT ($F_{1, 24} = 5.16$, $p = 0.032$; Fig. 6D). Similarly, the expression level of *Dmrt1* was higher at MPT than at FPT from stage 16 onwards, with a peak at stage 19 ($F_{1, 24} = 68.74$, $p < 0.001$; Fig. 6E). Inconsistent with the expression levels of the other genes, the expression level of *Sox9* did not differ between MPT and FPT throughout the presumptive TSP ($F_{1, 24} = 1.04$, $p = 0.31$; Fig. 6F).

```
Mauremys    MNLLDPFMKM TDEQDKCISG APSPTMSDDS AGSPCPSGSG SDTENTRPQE NTFPKGDPDL KKESDEDKFP VCIREAVSQV LKGYDWTLVP MPVRVNGSSK [100]
Trachemys   .......... .E........ .......... .......... .......... .......... .......... .......... .......... .......... [100]
Alligator   .......... .E..E..... .......... .......... .......... .......... ..D....... .......... .......... .......... [100]
Gallus      .......... .E.....D.. .......... .......... .......... .......... .......... .......... .......... .......... [100]
Mus         .......... ....E.GL.. .......E.. .......... .......... .......... .E........ ....E..... .......... .......... [100]
Homo        .......... ....E.GL.. .......E.. .......... .......... .......... .E........ ....E..... .......... .......... [100]

Mauremys    NKPHVKRPMN AFMVWAQAAR RKLADQYFHL HNAELSKTLG KLWRLLNESE KRPFVEEAER LRVQHKKDHP DYKYQPRRRK SVKNGQSEQE EGSEQTHISP [200]
Trachemys   ....|..... .......... .......... .......... .......... .......... .......... ...|...... .......... .......... [200]
Alligator   ....|..... .......... .......... .......... .......... .......... .......... ...|...... .......... .......... [200]
Gallus      ....|..... .......... .......... .......... .......... .......... .......... ...|...... .......... .......... [200]
Mus         ....|..... .......... .......... .......... .......... .......... .......... ...|...... .A..A...AT...... [200]
Homo        ...|...... .......... .......... .......... .......... .......... .......... ...|...... .A..A...AT...... [200]

Mauremys    NAIFKALQAD SPQSSSSMSE VHSPGEHSGQ SQGPPTPPTT PKTDVQ-PGK QDLKREGRPL QEGGRQPPHI DFRDVDIGEL SSDVISNIET FDVNEFDQYL [300]
Trachemys   .......... .......... .......... .......... ......-... .......... .......... .......... .......... .......... [300]
Alligator   .......... .......... .......... .......... ......-... .......... .......... .......... .......... .......... [300]
Gallus      .......... ........I. .......... .......... ....A.Q... .......... A......... .......... .......... .......... [300]
Mus         .......... ..H...G... .......... .......... ......-... A......... P........-. .......... .......... .......... [300]
Homo        .......... ..H...G... .......... .......... .....-A. V......... A......-. .......... .......... .......... [300]

Mauremys    PPNGHPGVPA THGQPGQVTY SGSYGISSTS ATQAGAGHVW MAKQ---QPQ PQQ----PPP PQAQPQ---- ---------- --PQHTMTTL SSEQGQSQQR [400]
Trachemys   .......... .......... .......... .......P.. .S.. ---P. ...Q---. .. ...P.. ---- ---------- --.P...... .......... [400]
Alligator   .......... .......... T........A ..PT...... .S.. ---PQP ...PQPPQ.. .... .. ---- ---------- --AA....P. .G...... [400]
Gallus      .......... ....--VT.. ..T....SA SSP.....A. ...---... .P.. ---... .P...P---- ---------- --A..LPA. .G...PA... [400]
Mus         .......... ....---... T........A ..P.S..... .S..QAPP.P ...PPQA..A ...P..PQAA PPQQPAAPPQ QPQA..L... ...P....-. [400]
Homo        .......... ....---... T........A P.P.T..... .S..QAPP.P ...PPQA.QA ...P..-QQA PPQQPQAP-Q QQQA..L... ...P....-. [400]

Mauremys    THIKTEQLSP SHYSEQQQHS PQQ------- LNYSSFNLQH YSSSYPTITR SQYDYTDHQS SNSYYSHAAS QSTSLYSTFT YMNPTQRPMY TPIADTTGVP [500]
Trachemys   .......... .......... ...------- ....P..... .......... .......... .......... ......F.... ....A..... .......... [500]
Alligator   P......... .......... ...------- I......... .......... .......... ...G...... .......... .......... ......S... [500]
Gallus      P......... .......... ...QQQQQQQ .G.G...... .G...P... ......E.N .G......G .GG...... .......... ......S... [500]
Mus         .......... .......... ...------- IA..P...P. ..P..P... ......N .S.......G .G.G...... .......A.. .......S... [500]
Homo        .......... .......... ...------- IS..P...P. ..P..P... ....A...N .G......G .GSG...... .......A.. .......S... [500]

Mauremys    SIPQTHSPQH WEQPVYTQLT RP [522]
Trachemys   .......... .......... .. [522]
Alligator   .......... .......... .. [522]
Gallus      .......... .......... .. [522]
Mus         .......... .......... .. [522]
Homo        .......... .......... .. [522]
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Fig. 5. Deduced amino acid sequence alignment for turtle, alligator, chicken, mouse, and human SOX9. The box demarcates the conserved domain of the SOX-TCF HMG-box. Accession numbers for the sequences used are turtle (*Trachemys scripta*), ACG70782; alligator (*Alligator mississippiensis*), NP001274197; chicken (*Gallus gallus*), NP989612; mouse (*Mus musculus*), NP035578; human (*Homo sapiens*), NP000337.

Discussion

We obtained full-length ORFs of 5 genes that are candidates involved in the sex determination cascade of *M. reevesii* given their role in this developmental process in other vertebrates. In addition, the amino acid sequences were characterized. This information will help us to understand the functions of the gene products and provide a basis for further studies on how these genes have evolved. A recent study showed that a consistent amino acid shift at positions 54 and 57 in DMRT1 distinguished GSD and TSD species and accompanied the evolution of GSD in reptiles from an ancestral TSD condition, as turnover events occurred by 1 or 2 mutations at least 3 times among non-avian reptiles [Janes et al., 2014]. Thus, our study contributes in identifying amino acid sequences of other sex-related genes in additional reptiles, which should help to understand whether the inter-species difference in protein sequences accompanies the shift between TSD and GSD and to illuminate the evolution of sex determination mechanisms.

Our study also uncovered the AKG expression patterns of these genes during the presumptive TSP in *M. reevesii* and points to their potential roles in urogenital development and perhaps sex determination and differentiation. On one hand, *Foxl2*, *Rspo1,* and *Cyp19a1* are genes with female-biased expression involved in ovarian formation in vertebrates, and consistently, these genes show sex-dimorphic expression during the presumptive TSP, with significantly higher expression levels at FPT than at MPT in *M. reevesii* (Fig. 6A–C). In contrast, *Sf1* and *Dmrt1* are involved in testicular development in ver-

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Fig. 6. Effects of temperature on the expressions of *Cyp19a1*, *Foxl2*, *Rspo1*, *Sf1*, *Dmrt1*, and *Sox9* in the adrenal-kidney-gonad (AKG) complex (*n* = 3 mixed samples) of *Mauremys reevesii* using quantitative PCR. Relative transcript abundance was normalized to the constitutively expressed gene β-actin. Expression levels at the female-producing temperature (FPT) are shown in open circles, levels at the male-producing temperature (MPT) are shown in black circles. The *y*-axis indicates fold changes of gene expression with respect to expression during stage 15 at MPT. The *x* axis indicates the developmental stage. Asterisks indicate significant difference between temperatures. Factorial ANOVA showed that temperature significantly affected the expression levels of the genes ($p < 0.0001$). The data are presented as mean fold changes ± standard error.
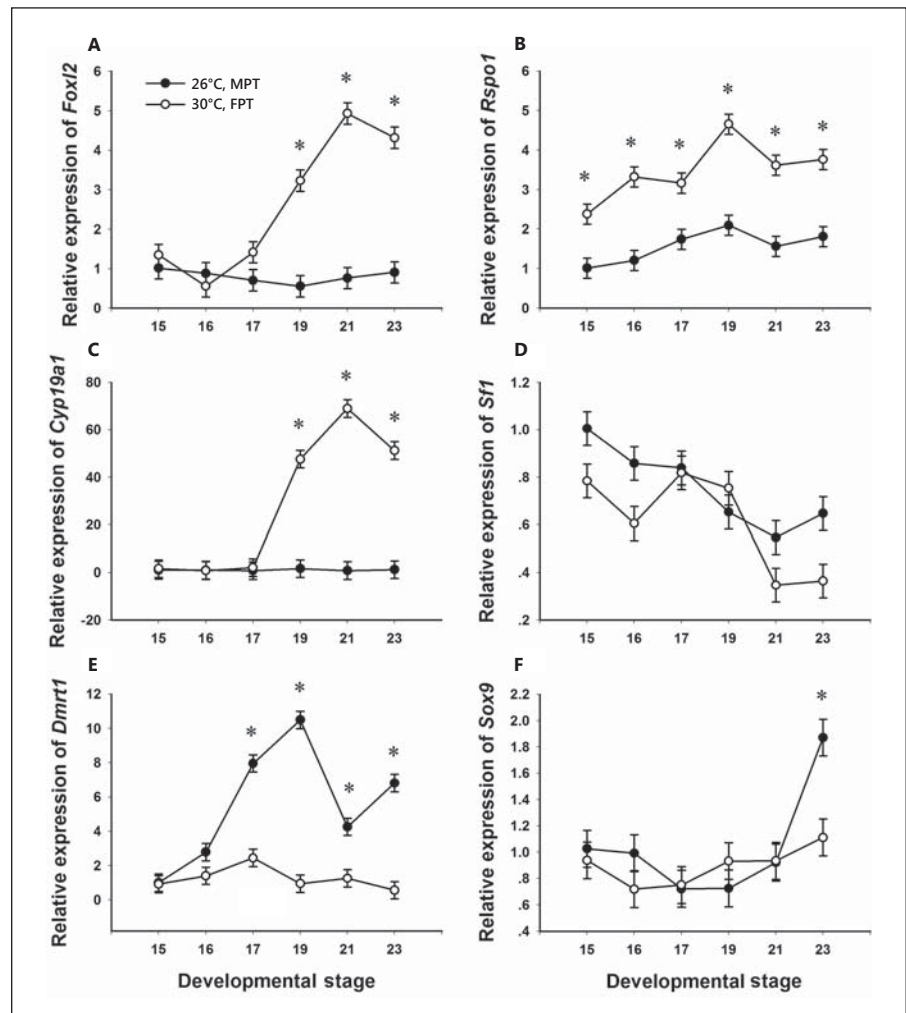
tebrates, and here they showed male-biased expression (Fig. 6D, E). As in other turtles, *Sox9* expression was male-biased only at the end of the presumptive TSP [Torres Maldonado et al., 2002; Shoemaker et al., 2007b], which is different from the expression pattern found in the developing gonads of mammals, perhaps because we examined AKG complexes and not gonads alone. Below, we discuss the roles of these genes in sex determination and differentiation in greater detail.

Genes with Female-Biased Expression

The 3 female-biased genes, *Foxl2*, *Rspo1,* and *Cyp19a1,* play an important role in female sexual development.

Foxl2 is a member of the forkhead-box transcription factor family, which is a key factor in female sex determination in both GSD and TSD. *Foxl2* deficiency causes premature ovarian failure and the blepharophimosis-

ptosis-epicanthus inversus syndrome [Crisponi et al., 2001; Loffler et al., 2003]. In mammals, *Foxl2* is required to suppress ovary-to-testis transdifferentiation. Loss of *Foxl2* expression in the follicle leads to upregulation of *Sox9*, and follicle and theca cells reprogram to form Sertoli and Leydig cells [Uhlenhaut et al., 2009]. The *Foxl2* female-biased expression observed in our study mimics the pattern in other TSD reptiles [Matsumoto and Crews, 2012; Janes et al., 2013].

Rspo1 is located upstream in the Wnt pathway and is involved in early gonadal development [Bernard et al., 2012; Chassot et al., 2012]. *Rspo1* deficiency can lead to female-to-male sex reversal in mammals [Parma et al., 2006; Chassot et al., 2008]. Consistent with this role, *Rspo1* was found to be upregulated in our study during female development as in other TSD reptiles (Fig. 6B) [Smith et al., 2008] and fish [Zhang et al., 2011; Zhou et al., 2016].

Cyp19a1 is involved in various developmental and biochemical processes (e.g., secondary sexual characteristics, hormone secretion, and glucose homeostasis) in GSD vertebrates [Rigaudiere et al., 1989; Baghaei et al., 2003; Bader et al., 2012; Chen et al., 2015]. Aromatase, a gene product of *Cyp19a1*, is a key enzyme for estrogen biosynthesis [Kitano et al., 1999] and is a key factor in sex determination and ovarian differentiation [Bogart, 1987; Lambeth et al., 2013], because estrogen, the product of the aromatization of testosterone by aromatase, can override the effect of male-determining genes (e.g., *Sry*) and induce ovarian development [Kobayashi et al., 2003; Pask et al., 2010]. In some fish and reptiles, *Cyp19a1* shows female-biased expression, as found in our study (fig. 6C) [Gabriel et al., 2001; Ramsey et al., 2007], and environmental temperatures can affect *Cyp19a1* expression and in turn reverse the sex of GSD teleosts and TSD reptiles [Baroiller et al., 2009; Matsumoto and Crews, 2012]. In addition, the methylation level of *Cyp19a1* is highly correlated with its expression and temperature [Matsumoto et al., 2013, 2016]. Therefore, *Cyp19a1* appears to play a critical role in sex differentiation of TSD species.

Genes with Male-Biased Expression
Sf1 and *Dmrt1* show significantly male-biased expression in *M. reevesii* (Fig. 6D, E) as well as in some other species.

Sf1 encodes a nuclear orphan receptor involved in the formation of steroidogenic organs and in turn in sex determination [Parker and Schimmer, 1997; Morohashi, 1999]. The expression pattern of *Sf1* is not conserved among different lineages of vertebrates [Valenzuela et al., 2013]. It is highly expressed during testicular development in mammals [Hatano et al., 1994; Ikeda et al., 1994] and during ovarian development in birds and fish [Smith et al., 1999; Ijiri et al., 2008]. Similarly, the expression pattern of *Sf1* differs among reptiles with TSD. For example, *Sf1* is expressed in a female-biased way in *A. mississippiensis* [Western et al., 2000] but male-biased in turtles (*M. reevesii, T. scripta,* and *C. picta*) [Fleming et al., 1999; Crews et al., 2001; Valenzuela et al., 2013; this study]. Although in our result *Sf1* is more highly expressed in the entire AKG complex at MPT than at FPT, the dimorphic expression across stages differed from the pattern observed in other TSD turtles. Because *Sf1* is highly expressed in the adrenal, this could mask subtle differential expression in the gonad that might exist [Fleming et al., 1999], as observed in other TSD turtles [Fleming and Crews, 2001; Valenzuela et al., 2006, 2013].

Dmrt1 encodes a putative transcription factor containing a zinc-finger-like DNA-binding motif (DM domain) [Shen and Hodgkin, 1988], and its homologs have been found in all vertebrates [Raymond et al., 1999; Nanda et al., 2002; Koopman, 2009]. *Dmrt1,* or its homolog *dmy,* plays an important role in sex determination of birds [Koopman, 2009] and fish [Matsuda et al., 2002]. Similarly, our study indicated that *Dmrt1* may also be responsible for male sex determination in *M. reevesii* because of its male-biased expression during the early presumptive TSP (Fig. 6E), which is consistent with the findings of previous studies on other TSD reptiles [Murdock and Wibbels, 2006; Shoemaker et al., 2007b].

Sox9 is an important sex determination gene in mammals, because it is a direct target of *SRY* and can upregulate the down-stream target *Amh*, which leads to mullerian duct degeneration [Hiramatsu et al., 2010]. However, in *Xenopus tropicalis*, *Sox9* is regarded as a gene involved in sex differentiation rather than sex determination, because the expression of *Sox9* is first detectable when the gonads are well differentiated [El Jamil et al., 2008]. Our study detected monomorphic *Sox9* expression from stage 15 to 22 and dimorphic male-biased expression at the end of the presumptive TSP, suggesting that *Sox9* may be involved in late testis differentiation as previously shown in other turtles [Torres Maldonado et al., 2002; Shoemaker et al., 2007b; Diaz-Hernandez et al., 2012]. Alternatively, our use of AKG complexes could have masked earlier gonadal differential expression of *Sox9* as it has been reported in other TSD turtle studies [Shoemaker et al., 2007b; Valenzuela et al., 2013]. Further, in *L. olivacea*, *Sox9* is expressed in medullary cords of the bipotential gonads in both male and female embryos and is turned off in presumptive ovaries and remains expressed in presumptive testis [Diaz-Hernandez et al., 2012], a pattern that differs from other TSD turtles [Valenzuela et al., 2013]. However, discrepancies between our study and findings in other vertebrates may represent true interspecific differences in *Sox9* expression, which is known to be evolutionarily labile [Valenzuela et al., 2013]. For instance, a pattern similar to ours was found in *A. mississippiensis* [Western et al., 1999b], but a male-biased expression pattern was detected in some turtles (*L. olivacea* and *T. scripta*) [Torres Maldonado et al., 2002; Shoemaker et al., 2007b]. In addition, SRY along with SF1 binds to the enhancer of *Sox9* in mammals [Sekido and Lovell-Badge, 2008], while in TSD turtles which lack *SF1*, the regulation of *Sox9* is certainly different than in mammals.

In conclusion, vertebrate ovarian developmental genes *Cyp19a1*, *Foxl2,* and *Rspo1* exhibit consistent expression

patterns in the developing AKG complexes of *M. reevesii*, whereas the expression of *Sf1*, *Dmrt1*, and *Sox9* is consistent with their involvement in the male developmental pathway. However, whether discrepancies in the observed profiles for *Sf1* and *Sox9* are attributable to their masking expression in adrenal-kidney tissue remains uncertain. Taken together, these genes are important candidates involved in sex determination and differentiation in *M. reevesii,* and further research using gonadal tissue separated from the adrenal-kidney is warranted. Our study provides a solid basis for further investigations on the molecular mechanism of sex determination in this species as well as other vertebrates in general.

Statement of Ethics

The research was performed under approval of the Animal Ethics Committee at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

Disclosure Statement

The authors have no conflicts of interest to declare.

References

Bader MI, Reitmayer C, Arndt C, Wober J, Kretzschmar G, et al: Age dependency of estrogen responsiveness in the uterus and adipose tissue of aromatase-knockout (ArKO) mice. J Steroid Biochem Mol Biol 128:29–37 (2012).

Baghaei F, Rosmond R, Westberg L, Hellstrand M, Eriksson E, et al: The *CYP19* gene and associations with androgens and abdominal obesity in premenopausal women. Obes Res 11:578–585 (2003).

Baroiller JF, D'Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G: Tilapia sex determination: where temperature and genetics meet. Comp Biochem Physiol Part A Mol Integr Physiol 153:30–38 (2009).

Bernard P, Ryan J, Sim H, Czech DP, Sinclair AH, et al: Wnt signaling in ovarian development inhibits Sf1 activation of *Sox9* via the *Tesco* enhancer. Endocrinology 153:901–912 (2012).

Bieser KL, Wibbels T: Chronology, magnitude and duration of expression of putative sex-determining/differentiation genes in a turtle with temperature-dependent sex determination. Sex Dev 8:364–375 (2014).

Bogart MH: Sex determination: a hypothesis based on steroid ratios. J Theor Biol 128:349–357 (1987).

Bull JJ: Sex determination in reptiles. Q Rev Biol 55:3–21 (1980).

Bull JJ, Vogt RC: Temperature-sensitive periods of sex determination in Emydid turtles. J Exp Zool 218:435–440 (1981).

Bull JJ, Hillis DM, O'Steen S: Mammalian ZFY sequences exist in reptiles regardless of sex-determining mechanism. Science 242:567–569 (1988).

Bullejos M, Koopman P: Spatially dynamic expression of *Sry* in mouse genital ridges. Dev Dyn 221:201–205 (2001).

Capel B, Tanaka M: Forward to the special issue on sex determination. Dev Dyn 242:303–306 (2013).

Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, et al: Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. Hum Mol Genet 17:1264–1277 (2008).

Chassot AA, Bradford ST, Auguste A, Gregoire EP, Pailhoux E, et al: WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. Development 139:4461–4472 (2012).

Chen Z, Wang O, Nie M, Elison K, Zhou D, et al: Aromatase deficiency in a Chinese adult man caused by novel compound heterozygous *CYP19A1* mutations: effects of estrogen replacement therapy on the bone, lipid, liver and glucose metabolism. Mol Cell Endocrinol 399:32–42 (2015).

Crews D, Fleming A, Willingham E, Baldwin R, Skipper JK: Role of steroidogenic factor 1 and aromatase in temperature-dependent sex determination in the red-eared slider turtle. J Exp Zool 290:597–606 (2001).

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, et al: The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166 (2001).

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

Desvages G, Pieau C: Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. J Steroid Biochem Mol Biol 41:851–853 (1992).

Diaz-Hernandez V, Marmolejo-Valencia A, Harfush M, Merchant-Larios H: Formation of the genital ridges is preceded by a domain of ectopic Sox9-expressing cells in *Lepidochelys olivacea*. Dev Biol 361:156–166 (2012).

DiNapoli L, Capel B: SRY and the standoff in sex determination. Mol Endocrinol 22:1–9 (2008).

Du WG, Hu LJ, Lu JL, Zhu LJ: Effects of incubation temperature on embryonic development rate, sex ratio and post-hatching growth in the Chinese three-keeled pond turtle, *Chinemys reevesii*. Aquaculture 272:747–753 (2007).

Du WG, Wang L, Shen JW: Optimal temperatures for egg incubation in two Geoemydid turtles: *Ocadia sinensis* and *Mauremys mutica*. Aquaculture 305:138–142 (2010).

El Jamil A, Kanhoush R, Magre S, Boizet-Bonhoure B, Penrad-Mobayed M: Sex-specific expression of *SOX9* during gonadogenesis in the amphibian *Xenopus tropicalis*. Dev Dyn 237:2996–3005 (2008).

Endo D, Kanaho Y, Park MK: Expression of sex steroid hormone-related genes in the embryo of the leopard gecko. Gen Comp Endocrinol 155:70–78 (2008).

Ferguson MW, Joanen T: Temperature of egg incubation determines sex in *Alligator mississippiensis*. Nature 296:850–853 (1982).

Fleming A, Crews D: Estradiol and incubation temperature modulate regulation of steroidogenic factor 1 in the developing gonad of the red-eared slider turtle. Endocrinology 142:1403–1411 (2001).

Fleming A, Wibbels T, Skipper JK, Crews D: Developmental expression of steroidogenic factor 1 in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 116:336–346 (1999).

Gabriel WN, Blumberg B, Sutton S, Place AR, Lance VA: Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. J Exp Zool 290:439–448 (2001).

Gomez-Saldarriaga C, Valenzuela N, Ceballos CP: Effects of incubation temperature on sex determination in the endangered Magdalena river turtle, *Podocnemis lewyana*. Chelonian Conserv Bi 15:43–53 (2016).

Greenbaum E: A standardized series of embryonic stages for the emydid turtle *Trachemys scripta*. Can J Zool 80:1350–1370 (2002).

Hatano O, Takayama K, Imai T, Waterman MR, Takakusu A, et al: Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 120:2787–2797 (1994).

Hiramatsu R, Harikae K, Tsunekawa N, Kurohmaru M, Matsuo I, Kanai Y: FGF signaling directs a center-to-pole expansion of tubulogenesis in mouse testis differentiation. Development 137:303–312 (2010).

Hoshi H, Nakao A: Molecular cloning of full-length Dmrt1 cDNA of Reeves turtle (*Chinemys reevesii*). J Vet Med Sci 70:687–692 (2008).

Ijiri S, Kaneko H, Kobayashi T, Wang DS, Sakai F, et al: Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 78:333–341 (2008).

Ikeda Y, Shen WH, Ingraham HA, Parker KL: Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Mol Endocrinol 8:654–662 (1994).

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.). Gen Comp Endocrinol 220:55–60 (2015).

Janes DE, Elsey RM, Langan EM, Valenzuela N, Edwards SV: Sex-biased expression of sex-differentiating genes *FOXL2* and *FGF9* in American alligators, *Alligator mississippiensis*. Sex Dev 7:253–260 (2013).

Janes DE, Organ CL, Stiglec R, O'Meally D, Sarre SD, et al: Molecular evolution of *Dmrt1* accompanies change of sex-determining mechanisms in reptilia. Biol Lett 10:20140809 (2014).

Janzen FJ, Paukstis GL: Environmental sex determination in reptiles: ecology, evolution, and experimental design. Q Rev Biol 66:149–179 (1991).

Kashimada K, Koopman P: Sry: the master switch in mammalian sex determination. Development 137:3921–3930 (2010).

Kitano T, Takamune K, Kobayashi T, Nagahama Y, Abe SI: Suppression of *P450* aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol 23:167–176 (1999).

Kobayashi T, Kajiura-Kobayashi H, Nagahama Y: Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. Cytogenet Genome Res 101:289–294 (2003).

Koopman P: Sex determination: the power of *DMRT1*. Trends Genet 25:479–481 (2009).

Lambeth LS, Cummins DM, Doran TJ, Sinclair AH, Smith CA: Overexpression of aromatase alone is sufficient for ovarian development in genetically male chicken embryos. PLoS One 8:e68362 (2013).

Loffler KA, Zarkower D, Koopman P: Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: *FOXL2* is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144:3237–3243 (2003).

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, et al: *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559–563 (2002).

Matsumoto Y, Crews D: Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Mol Cell Endocrinol 354:103–110 (2012).

Matsumoto Y, Buemio A, Chu R, Vafaee M, Crews D: Epigenetic control of gonadal aromatase (*cyp19a1*) in temperature-dependent sex determination of red-eared slider turtles. PLoS One 8:e63599 (2013).

Matsumoto Y, Hannigan B, Crews D: Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (*cyp19a1*) gene in species with temperature-dependent sex determination. PLoS One 11:e0167362 (2016).

Mitchell NJ, Nelson NJ, Cree A, Pledger S, Keall SN, Daugherty CH: Support for a rare pattern of temperature-dependent sex determination in archaic reptiles: evidence from two species of tuatara (Sphenodon). Front Zool 3:9 (2006).

Morohashi K: Gonadal and extragonadal functions of Ad4BP/SF-1: developmental aspects. Trends Endocrinol Metab 10:169–173 (1999).

Murdock C, Wibbels T: Dmrt1 expression in response to estrogen treatment in a reptile with temperature-dependent sex determination. J Exp Zool B Mol Dev Evol 306:134–139 (2006).

Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, et al: Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. Genetics 191:163–170 (2012).

Nanda I, Kondo M, Hornung U, Asakawa S, Winkler C, et al: A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. Proc Natl Acad Sci USA 99:11778–11783 (2002).

Parker KL, Schimmer BP: Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 18:361–377 (1997).

Parma P, Radi O, Vidal V, Chaboissier MC, Dellambra E, et al: R-spondin1 is essential in sex determination, skin differentiation and malignancy. Nat Genet 38:1304–1309 (2006).

Pask AJ, Calatayud NE, Shaw G, Wood WM, Renfree MB: Oestrogen blocks the nuclear entry of SOX9 in the developing gonad of a marsupial mammal. BMC Biol 8:113 (2010).

Radhakrishnan S, Literman R, Neuwald J, Severin A, Valenzuela N: Transcriptomic responses to environmental temperature by turtles with temperature-dependent and genotypic sex determination assessed by RNAseq inform the genetic architecture of embryonic gonadal development. PLoS One 12:e0172044 (2017).

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 75:978–991 (2007).

Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ, Zarkower D: Expression of *Dmrt1* in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev Biol 215:208–220 (1999).

Rhen T, Crews D: Distribution of androgen and estrogen receptor mRNA in the brain and reproductive tissues of the leopard gecko, *Eublepharis macularius*. J Comp Neurol 437:385–397 (2001).

Rigaudiere N, Grizard G, Boucher D: Aromatase activity in purified Leydig cells from adult rat. Comparative effects of insulin, IGF-I, and hCG. Acta Endocrinol (Copenh) 121:677–685 (1989).

Schroeder AL, Metzger KJ, Miller A, Rhen T: A novel candidate gene for temperature-dependent sex determination in the common snapping turtle. Genetics 203:557–571 (2016).

Sekido R, Lovell-Badge R: Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer. Nature 456:824–824 (2008).

Shen MM, Hodgkin J: *mab-3*, a gene required for sex-specific yolk protein expression and a male-specific lineage in *C. elegans*. Cell 54:1019–1031 (1988).

Shoemaker CM, Queen J, Crews D: Response of candidate sex-determining genes to changes in temperature reveals their involvement in the molecular network underlying temperature-dependent sex determination. Mol Endocrinol 21:2750–2763 (2007a).

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

Smith CA, Sinclair AH: Sex determination in the chicken embryo. J Exp Zool 290:691–699 (2001).

Smith CA, Elf PK, Lang JW, Joss JM: Aromatase enzyme-activity during gonadal sex-differentiation in alligator embryos. Differentiation 58:281–290 (1995).

Smith CA, Smith MJ, Sinclair AH: Gene expression during gonadogenesis in the chicken embryo. Gene 234:395–402 (1999).

Smith CA, Shoemaker CM, Roeszler KN, Queen J, Crews D, Sinclair AH: Cloning and expression of *R-Spondin1* in different vertebrates suggests a conserved role in ovarian development. BMC Dev Biol 8:72 (2008).

Torres Maldonado LC, Landa Piedra A, Moreno Mendoza N, Marmolejo Valencia A, Meza Martinez A, Merchant Larios H: Expression profiles of *Dax1*, *Dmrt1*, and *Sox9* during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. Gen Comp Endocrinol 129:20–26 (2002).

Udar N, Yellore V, Chalukya M, Yelchits S, Silva-Garcia R, et al: Comparative analysis of the *FOXL2* gene and characterization of mutations in BPES patients. Hum Mut 22:222–228 (2003).

Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, et al: Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. Cell 139:1130–1142 (2009).

Urushitani H, Katsu Y, Miyagawa S, Kohno S, Ohta Y, et al: Molecular cloning of anti-Mullerian hormone from the American alligator, *Alligator mississippiensis*. Mol Cell Endocrinol 333:190–199 (2011).

Valenzuela N: Temperature-dependent sex determination, in Deeming DC (ed): Reptilian Incubation: Environment, Evolution and Behavior, pp 211–227 (Nottingham University Press, Nottingham 2004).

Valenzuela N: Evolution of the gene network underlying gonadogenesis in turtles with temperature-dependent and genotypic sex determination. Integr Comp Biol 48:476–485 (2008a).

Valenzuela N: Relic thermosensitive gene expression in a turtle with genotypic sex determination. Evolution 62:234–240 (2008b).

Valenzuela N, LeClere A, Shikano T: Comparative gene expression of steroidogenic factor 1 in *Chrysemys picta* and *Apalone mutica* turtles with temperature-dependent and genotypic sex determination. Evol Dev 8:424–432 (2006).

Valenzuela N, Neuwald JL, Literman R: Transcriptional evolution underlying vertebrate sexual development. Dev Dyn 242:307–319 (2013).

Viets BE, Tousignant A, Ewert MA, Nelson CE, Crews D: Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. J Exp Zool 265:679–683 (1993).

Western PS, Harry JL, Graves JA, Sinclair AH: Temperature-dependent sex determination in the American alligator: *AMH* precedes *SOX9* expression. Dev Dyn 216:411–419 (1999a).

Western PS, Harry JL, Graves JA, Sinclair AH: Temperature-dependent sex determination: upregulation of *SOX9* expression after commitment to male development. Dev Dyn 214:171–177 (1999b).

Western PS, Harry JL, Marshall Graves JA, Sinclair AH: Temperature-dependent sex determination in the American alligator: expression of *SF1*, *WT1* and *DAX1* during gonadogenesis. Gene 241:223–232 (2000).

Wilhelm D, Palmer S, Koopman P: Sex determination and gonadal development in mammals. Physiol Rev 87:1–28 (2007).

Willingham E, Baldwin R, Skipper JK, Crews D: Aromatase activity during embryogenesis in the brain and adrenal-kidney-gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Gen Comp Endocrinol 119:202–207 (2000).

Yatsu R, Miyagawa S, Kohno S, Parrott BB, Yamaguchi K, et al: RNA-seq analysis of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent sex determination and differentiation. BMC Genomics 17:77 (2016).

Zhang YM, Li F, Sun DC, Liu JD, Liu N, Yu QX: Molecular analysis shows differential expression of R-spondin1 in zebrafish (*Danio rerio*) gonads. Mol Biol Rep 38:275–282 (2011).

Zhou L, Charkraborty T, Zhou Q, Mohapatra S, Nagahama Y, Zhang Y: Rspo1-activated signalling molecules are sufficient to induce ovarian differentiation in XY medaka (*Oryzias latipes*). Sci Rep 6:19543 (2016).