Multivariate Expression Analysis of the Gene Network Underlying Sexual Development in Turtle Embryos with Temperature-Dependent and Genotypic Sex Determination

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Abstract

Sexual development has long been the target of study and despite great advances in our understanding of the composition and regulation of the gene network underlying gonadogenesis, our knowledge remains incomplete. Of particular interest is the relative role that the environment and the genome play in directing gonadal formation, especially the effect of environmental temperature in directing this process in vertebrates. Comparative analyses in closely related taxa with contrasting sex-determining mechanisms should help fill this gap. Here I present a multivariate study of the regulation of the gene network underlying sexual development in turtles with temperature-dependent (TSD; *Chrysemys picta*) and genotypic sex determination (GSD; *Apalone mutica*). I combine novel data on *SOX9* and *DMRT1* from these species with contrasting sex-determining mechanisms for the first time with previously reported data on *DAX1*, *SF-1* (*NR5A1*), *WT1*, and *aromatase (CYP19A1)* from these same taxa. Comparative expression analyses of *SOX9* and *DMRT1* from these and other species indicate additional elements whose expression has diverged among TSD taxa, further supporting the notion that significant evolutionary changes have accrued in the regulation of the TSD gene network in reptiles. A non-parametric MANOVA revealed that temperature had a significant effect in multivariate gene expression in *C. picta* that varied during embryonic development, whereas the covariation of gene expression in *A. mutica* was insensitive to temperature. A phenotypic trajectory analysis (PTA) of gene expression comparing both species directly indicated that the relative covariation in gene expression varied between temperatures in *C. picta*. Furthermore, the 25°C trajectory of *C. picta* differed from that of *A. mutica* in the magnitude of gene expression change. Additional analyses revealed a stronger covariation in gene expression and a more interconnected regulatory network in *A. mutica*, consistent with the hypothesis that sexual development is a more canalized process in *A. mutica*, as would be expected if GSD evolved in this lineage through directional selection from its TSD ancestor.

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Sexual development is a textbook case of a well-known developmental paradigm. In animals, the genome is most often the provider of instructions for the development of structures and the environment is permissive (its role is

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to support and not disturb this process), while in other cases the environment provides the instructions of what structures to make and the genome is permissive and has to simply provide the genetic tools to build them [Gilbert and Epel, 2009]. Genotypic (GSD) and environmental (ESD) sex-determining mechanisms are a perfect example of such role-reversal between genome and environment in the case of sexual development. In the most common ESD mechanism found in vertebrates it is the environmental temperature during embryogenesis that plays the instructive role in gonadogenesis (temperature-dependent sex determination, TSD) [Valenzuela and Lance, 2004].

Reptiles are a particularly valuable group to study sexual development because they exhibit a wide range of sex-determining mechanisms, from strict GSD involving sex chromosomes to strict TSD and intermediate systems of GSD that are overridden by environmental effects in various degrees, thus spanning a continuum between potentially pure extremes [Valenzuela et al., 2003; Sarre et al., 2004; Valenzuela, 2008c]. Significant effort has been devoted to understanding this process of irreversible commitment to the male or female developmental fate in reptiles and how it can be initially triggered by genetic or environmental factors [Yao et al., 2004; Valenzuela, 2008c]. From a molecular perspective, the main focus has been to identify reptilian homologs of those genes known to be involved in sexual development in model systems [reviewed in Spotila et al., 1994; Place and Lance, 2004], typically studying the expression of one or a few genes at a time in a variety of species [Desvages and Pieau, 1992; Smith et al., 1995; Spotila et al., 1998; Fleming et al., 1999; Moreno-Mendoza et al., 1999; Western et al., 1999a; Gabriel et al., 2001; Place et al., 2001; Valenzuela et al., 2006; Valenzuela and Shikano, 2007]. This approach has been very successful in showing that gonadogenesis employs a gene regulatory network composed of numerous elements common to all vertebrates [reviewed in Place and Lance, 2004]. This approach has also resulted in a network model for TSD that is a chimerical combination of such fragmentary data. However, increasing efforts are now devoted to multigene comparisons in single taxa [e.g. Shoemaker et al., 2007a, b], which should be facilitated further by emerging genomic resources [Janes et al., 2008; Valenzuela, 2009b] and should provide species-specific network models needed for evolutionary and functional comparative genomics.

Although membership to this regulatory network has been conserved for many but not all elements studied (e.g., *SRY* is unique to therian mammals) [Wallis et al., 2008], this comparative research on non-model taxa has revealed significant divergence in the regulation of this network reflected in differences in expression patterns among species within and across TSD and GSD [e.g. Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a, b]. To explore the evolution of this network further, I present here a novel multivariate analysis of gene expression in TSD (painted turtles) and GSD (softshell turtles) reptiles combining new data from *SOX9* and *DMRT1* and previously reported expression data on other genes involved in gonadogenesis *(DAX1, WT1, aromatase (CYP19A1), and SF-1 (NR5A1))*, and discuss the covariation in the regulation of the gene network that underlies urogenital development in vertebrates.

SOX9 and *DMRT1*

The transcription factor *SOX9* is a member of a large family of *SOX* genes containing an *SRY*-like high-mobility group (HMG) box, and it is related to testis differentiation in mammals [Morrish and Sinclair, 2002]. *SOX9* is of particular interest in sex determination as it appears to be the direct downstream target of SRY in therian mammals. *SOX9* is a key partner along with *FGF9* of a reinforcing loop enabled by *SRY* which out-competes the action of an active female-promoting signal from *WNT4* (perhaps in conjunction with *RSPO1*) present in the bipotential gonad, thus tipping the balance toward male development [DiNapoli and Capel, 2008]. It is possible therefore that *SOX9* might be at the top of the developmental cascade for sex determination in species lacking *SRY* such as reptiles. *SOX9* is the first known marker of differentiating Sertoli cells in mammals where it has lifelong expression and it is also expressed in the epididymis and around the müllerian ducts in males (it regulates *AMH* expression), while it is not present in ovaries but is expressed in the metanephros of both sexes [Kent et al., 1996; Arango et al., 1999; Vidal et al., 2001; Clarkson and Harley, 2002; reviewed in Place and Lance, 2004]. The critical function of the HMG box occurs through its DNA-binding domain which attaches to the minor groove of the DNA, physically bending it and thus inducing contact between distant DNA sites or recruiting proteins unable to bind to DNA alone, consequently facilitating transcriptional control, gene recombination, or DNA repair [Ohe et al., 2002]. Additionally, the nuclear localization of SOX9 seems to be essential for its function in male sex determination such that the equilibrium of its nuclear import/export may regulate the repression of female- or

Table 1. Primers used for standard PCR or quantitative-real time PCR (those with qm prefix) amplification of *SOX9* and *DMRT1*, and β-actin cDNA fragments in *Chrysemys picta* and *Apalone mutica* (blank cells indicate that the same primers were used for both species because sequences were identical)

Primer	*Chrysemys picta* (5′–3′)	*Apalone mutica* (5′–3′)
β-actin-for	CAG GTC ATC ACC ATY GGC AA	
β-actin-rev	GCT TGC TGA TCC ACA TCT GC	
SOX9-for	CAG GAS AAA TGC ATC TCY GGC	
SOx9-rev	CTG NGC CCA CAC CAT GAA GG	
DMRT1-for	AAR AAG TGC AGC CTG ATC GC	
DMRT1-rev	CAT ATA TGT GGC TGG GAG GC	
qmβ-actin-for	AAG CCC TCT TCC AGC CAT	AAG CTC TCT TCC AGC CCT
qmβ-actin-rev	GAC AGC ACA GTG TTG GCG	
qm*SOX9-for*	AGA GAG CGA CGA GGA CAA ATT C	GAG AGC GAGG AGG ACA AGT TC
qm*SOX9-rev*	GGG TCC AGT CGT AAC CCT TCA	GTC CAG TCG TAG CCC TTC AG
qm*DMRT1*-for	ATC AGC CAT CCC ATC CCT	
qm*DMRT1*-rev	GTG TTG GGC TGC TGC TTT	GTG TCG GGC TGC TGC TTT

the activation of male-specific differentiation [Gasca et al., 2002]. Consistent with the mammalian model, *SOX9* expression is upregulated in male embryonic gonads in TSD turtles and crocodilians (*Trachemys scripta* [Spotila et al., 1998; Shoemaker et al., 2007a], *Lepidochelys olivacea* [Moreno-Mendoza et al., 1999; Torres-Maldonado et al., 2001], and alligators [Western et al., 1999b]) but notably, it is upregulated at female- rather than at male-producing temperatures in the TSD leopard gecko *Eublepharis macularius* [Valleley et al., 2001].

DMRT1 (doublesex and mab-3-related transcription factor 1) is another gene of great interest as it is a conserved regulator of sexual development in vertebrates [Morrish and Sinclair, 2002] and is a candidate sex-determining gene linked to the Z chromosome in birds [Nanda et al., 1999]. Like *SOX9*, *DMRT1* is a member of a gene family containing a conserved DNA-binding motif, the DM domain [Raymond et al., 1999], which also attaches to the DNA minor groove, but unlike *SOX9*, it does not induce DNA bending [Murphy et al., 2007]. *DMRT1* expression is higher in male than in female developing gonads of fish, bird, and mammalian embryos [Raymond et al., 1998; Smith et al., 1999; Kettlewell et al., 2000; Moniot et al., 2000]. In mammals, *DMRT1* upregulation in male gonads is observed after the appearance of the first gonadal sexual dimorphism [Yao and Capel, 2005]. Consistently, in TSD taxa *DMRT1* shows higher expression in male alligators [Smith et al., 1999] and *T. scripta* [Kettlewell et al., 2000; Shoemaker et al., 2007a] before sexual differentiation but after the onset of the thermosensitive period.

Methods

Gene Expression Data Collection and Analysis
Methods used here followed those described in Valenzuela et al. [2006], Valenzuela and Shikano [2007], and Valenzuela [2008a, b, 2009a, b], with some modification of the data analyses as presented below.

Sample Collection
Freshly laid eggs from 7 natural nests of *Chrysemys picta* and 17 nests of *Apalone mutica* [Valenzuela et al., 2006] were uniformly distributed and incubated at 25 and 30°C corresponding to male- and female-producing temperatures for *C. picta* [Ewert and Nelson, 1991], in boxes with moistened vermiculite set at –150 kPa. Incubation temperature does not bias sex ratios of *A. mutica* (GSD) [Janzen, 1993]. Humidity was maintained by replacing lost water (as determined by weight loss) prior to egg sampling or weekly otherwise, and boxes were rotated at least weekly within incubators. Embryos were collected at stages before (9 and 12), at the onset of (15), in the middle of (19), and at the end of (22) the thermosensitive period [sensu Yntema, 1968; Bull and Vogt, 1981]. Sampled embryos were stored in RNAlater at –20°C and subsequently at –80°C for later use.

Cloning and Quantitative PCR
RNA was extracted from the adrenal-kidney-gonadal (AKG) complex (whole embryos or trunks were used from stage 9 and 12 embryos, respectively) using Qiagen's RNeasy Kit, and DNAse-I digested. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer and its quality assessed by the presence of ribosomal bands in agarose gels stained with ethidium bromide. Individual samples were kept separate and analyzed without pooling. Total RNA (1 μg per sample) was retro-transcribed with (dT)$_{20}$ primers using Superscript III (Invitrogen). For samples that yielded less than 1 μg total RNA in 8 μl elute volume, as much as 8 μl were used for the RT-PCR, and the total amount of RNA was recorded for standardization during the analysis of data as described below.

Degenerate primers were designed from conserved regions of vertebrate *SOX9* and *DMRT1* cDNA sequences as found in Gen-Bank (table 1) and used to amplify a 315-bp *SOX9* and a 495-bp *DMRT1* cDNA fragment in both species, which were then cloned into a pGEM®-T Easy Vector System (Promega) and sequenced. A 343-bp β-actin *(ACTB)* cDNA fragment [Valenzuela et al., 2006] was used for RNA quality assessment during qPCR and for normalization of *SOX9* and *DMRT1* gene expression. Internal primers amplified a 124-bp β-actin fragment [Valenzuela et al., 2006], and a 73-bp and a 102-bp *SOX9* and *DMRT1* fragment, respectively (table 1), during real-time qPCR using Brilliant SYBR Green PCR Master Mix in an Mx3000P thermocycler (Stratagene). ROX was used as reference dye for background correction. Standard curves were generated from pure miniprep-cloned DNA obtained above by diluting it at concentrations of 5, 1, 1×10^{-2}, 1×10^{-4}, 1×10^{-6}, 1×10^{-12}, 1×10^{-14}, and 1×10^{-16} ng/μl, and run in duplicate in each qPCR to ensure technical repeatability of the results. Samples from all clutches, temperatures, and stages were included in each 96-well plate used for the qPCR experiments to avoid any systematic bias. qPCR conditions included: 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a dissociation-curve cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Dissociation profiles were inspected to confirm amplification of a single transcript.

Data Analysis
Initial template amount per sample and gene was calculated via standard-curve quantification using Mx3000p v2.0 from Stratagene with background correction. Initial copy numbers were calculated from the molecular weight of the fragments for each gene, and standardized to 1 μg of initial total RNA. Expression data for *SOX9* and *DMRT1* per individual were kept separate, normalized using β-actin expression data, and log$_2$ transformed to correct for heteroscedasticity and non-normality before statistical analysis [Valenzuela et al., 2006]. Significance of the temperature-treatment effect was determined by testing for differences in mean gene expression per developmental stage via an ANOVA using Jmp 7© [SAS Institute, 2004].

Several analyses were carried out to explore the multivariate covariation of gene expression of *SOX9*, *DMRT1* (this study), *DAX1* [Valenzuela, 2008a], *WT1* [Valenzuela, 2008b], *aromatase* [Valenzuela and Shikano, 2007], and *SF-1* [Valenzuela et al., 2006] as these studies employed the same set of embryos. Individuals with any missing data were excluded from these analyses. Sample sizes for multivariate analyses were n = 39 for *C. picta* and n = 75 for *A. mutica.* First, a full factorial non-parametric MANOVA [Anderson, 2001] was carried out in R 2.8.1 [R Core Development Team, 2008] for each species to test for the effect of temperature, developmental stage, and their interaction on combined gene expression. Second, a multivariate phenotypic trajectory analysis (PTA) was employed [Collyer and Adams, 2007; Adams and Collyer, 2009]. In this case, the expression profiles for all genes per embryo are the multivariate phenotype and their combination forms a trajectory of gene expression through developmental time. PTA was used to quantify and compare the multivariate gene expression trajectories through embryonic development between temperatures and species. Statistical significance was assessed via a randomization procedure with 9,999 iterations carried out in R 2.8.1 [R Core Development Team, 2008; Adams and Collyer, 2009]. Additionally, pairwise correlations across all stag-

es and temperatures were calculated in Jmp 7 to describe significant overall covariation in the expression of these genes within species and compared between taxa. Finally, a gene coexpression network for each species was estimated using first-order conditional independence based on partial correlation [Magwene and Kim, 2004]. Significance of the partial correlations (ρ) between gene pairs was assessed by calculating test values as

Test values = $-N \times \ln(1 - \rho^2)$

where N is the sample size, and compared to the critical value (3.84) of a χ^2 distribution with 1 df. Any partial correlations with a test value below 3.84 are non-significant and the edge connecting those 2 genes is removed from the network.

Results and Discussion

DMRT1 and SOX9 Cloning and Expression
The 495-bp *DMRT1* fragment sequenced here (accession numbers FJ791118 for *C. picta* and FJ791119 for *A. mutica*) was similar to *Crocodylus palustris* isoforms a1, a2, c, d, and e [Anand et al., 2008] and included the 3′ half of the DM domain box [Raymond et al., 1999]. Sequence identity between the *Chrysemys* fragment and that of other reptiles and chicken was high: 99% identical to *Trachemys,* 97% to *Mauremys*, 96% to *Apalone, Pelodiscus,* and *Lepidochelys,* 89% to *Crocodylus* and *Alligator*, 83% to *Gallus*, 82% to *Elaphe* and *Trimeresurus*, and 80% to *Calotes* and *Gekko.* The amino acid sequence was also similar to that of several other vertebrates, particularly at the DM box (fig. 1a).

Likewise, the *SOX9* fragment (accession numbers FJ804411 for *C. picta* and FJ804412 for *A. mutica*) (fig. 1b), which corresponds to a 5′ portion of the HMG box domain, showed high similarity to that of other vertebrates at the DNA level (99% identical to *Trachemys*, 93% to *Anas*, 90% to *Apalone*, 89% to *Alligator* and *Gallus*, 88% to *Eublepharis*, 86% to *Homo* and *Mus*, 84% to *Xenopus*, and 84% to *Calotes*) and the amino acid level (fig. 1b).

Standard curves for β-actin, *DMRT1,* and *SOX9* had R^2 values of 0.97–1.0 for *C. picta*, and of 0.98–0.997 for *A. mutica. DMRT1* and *SOX9* AKG expression in *A. mutica* and *C. picta* did not differ significantly between temperatures (fig. 2). The biological significance of differences in gene expression between these 2 species is presented in the multivariate analyses section, but some interesting contrasts among *C. picta* (fig. 2a, b) and other TSD taxa are described below.

DMRT1 expression levels in *C. picta* (fig. 2b) differed from those reported for *T. scripta* turtles by Kettlewell et al. [2000] using pooled AKGs and by Shoemaker et al. [2007a] for gonads. In *T. scripta, DMRT1* showed a grad-
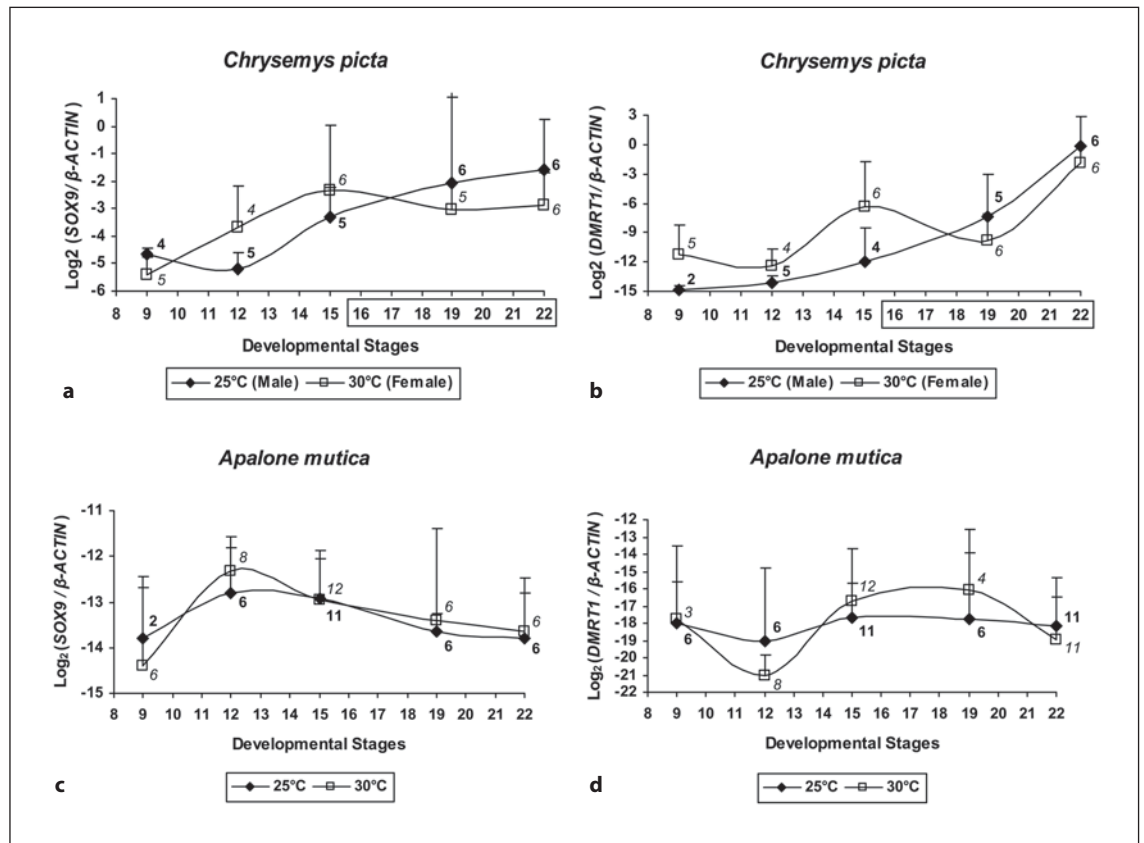
a. DMRT1

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50         60         70         80         90
Chrysemys     KKCSLIAERQ RVMAAQVALR RQQAQEEELG ISHPIPLPSA PELFVKKENN GGSSCLLLES SSPTHSTNT~ ATTASTTPSE GRMLIQDIPS
Apalone       .......... .......... .......... .......... .......... .......... ........~ .......S..A.
Trachemys     ~~~~~~~~~~ ~~~~~~~~~~ ~~.......  .......... .......... .......... ........~ ..........
Lepidochelys  ~~~~~~~~~~ ~~~~....  .......... .......... .......T.. .......N.. ........~ ..........
Pelodiscus    .......... .......... .......... .......... .......... .......... .......... .......S..A.
Crocodylus    .......... .......... .......... .......V... T..L.....S .......... .......S.~ V..V..S...
Mauremys      .......... .......... .......... .......... .......... ........N. .......... ..........
Alligator     ~~~....... .......... .......... .......... T.......... .......S.~ V..V..S... ...........V.
Trimeresurus  .......... .......... .......... T.MY..... AN.....~. T..PQ..T.~ ..VV..SST.
Elaphe        .......... .......... .......... T.MY..... AN.....~. T..PQ..T.~ ..VV..SST.
Gekko         ~~~~~~~~~~ ~~~~~~~~.~ .......... T.MYM..... ANG...... PQ..T.T SGAASST. ...M...
Calotes       .......... .......~~ ~.MY...S. ASGT..V..G G..PP..S.~ ..AG.SAAT. .....P.L..
Gallus        .......... ......V... .......... .V...... .PV...~SS SS.....QD. ...A..S.V AA.ASA.P.
Homo          ...N...... .......... .......... A..L..R.. .SNP..MT.C .GTSQPPP~~ .SVPT.AA.. ...V....A
Rattus        .......... .......... .......... A..L..R..S ASNP..MA.. ..SAQPPP~~ .S.PTP.V.. ...V....A
Mus           .......... .......... .......... A..L..R... ASNP..MA.N .SAQPPP~~ .S.PTPAA.. ...V....A
Xenopus       .......... .......... .....H..I. A..LI..HG .S...M..N ..TQTTST~~ P..SG..AS.. .KV...E...
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
                       100        110        120        130        140        150        160
Chrysemys     ITSRGHLEST SDLVVDSTYY SSFYQPSLYP YYNNLNYSP YQMAVASDSS SS~DMGGTLV GSPVKNSLRS LPATYM
Apalone       .......... .......... .......... ...Q..... ...E.... ...~..... .......... .......
Trachemys     .......... .......... .......... ...Q..... ...E.... ...~..... .......... .......
Lepidochelys  .......... .......... .......... ...Q..... ...E.... ...~..... .......... .......
Pelodiscus    .......... .......... .......... ...Q..... ...E.... ...~..... .......... .......
Crocodylus    .......... .......... .......... .......... ...E.. ~E...S.. ..........
Mauremys      ........A. ....... G......... ...Q..... ...E.. ..~EW.AHY. .........Q
Alligator     .......~. .......... .......... .......... ..........
Trimeresurus  LA........ ....M.P.. ..L...... ...Q..... ...TETT .G~...SP.T .....S... ..T...
Elaphe        LA........ ....M.P.. ..L...... ...Q..... ...TETT .G~...SP.S .....S.... ..T...
Gekko         LS........ .......... ...~... ...Q..... ...S.ETT .G~...SS... ...A......
Calotes       LS........ .......... .......... ...Q..... ...E.T .G~E..SP.. ..........
Gallus        .P........ .......... .......... .......TE.. ...~ET...F. .AM......
Homo          V.....V.N. P....S... .......F. ......CPQ .S..L..A..A .G~EV.NP.. .........G ..GP.V
Rattus        V.....M.NA P...MS.PA. .......F. .......PQ .S..LSAE.. .G~.V.NP.G .........P.V
Mus           V.....M.N. ...S.PA... .......F. .......PQ .S..LSAE.. .G~EV.NS.G .........P.V
Xenopus       ......M... ....M..P.. .N....P... .......P. ....M.AE.T .GN....~IS .P.L..NH.N H..A.V
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b. SOX9

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       10         20         30         40         50         60         70         80         90         100
Chrysemys     QEKCISGAPS PTMSDDSAGS PCPSGSGSDT ENTRPQENTF PKGD~PDLKK ESDEDKFPVC IREAVSQVLK GYDWTLVPMP VRVNGSSKNK PHVKRPMNAF MVWAQ
Apalone       .D........ .......... .......... ...R...A...E.....E........... .......... .......... ..........
Trachemys     .D........ .......... .......... .......... ..~..... .......... .......... .......... ..........
Alligator     .......... .......... .......... ...~... D.......... .......... .......... ..........
Calotes       ~~~~L..... ....E..... .......... ..S..Q... .......... .......F... ....R.... ..........
Eublepharis   ....L..... R......... ....V...A .N.~... .......... .......... .......... ..........
Gallus        .D....D... .......... .......... ..~..... .......... .......... .......... ..........
Homo          ...GL..... ....E..... .......... ..E~... ...E..... .......... .......... ..........
Mus           ...GL..... ....E..... .......... ..E~... ...E..... .......... .......... ..........
Anas          .D....D... .......... .......... ..~..... .......... .......... .......... ..........
Xenopus       .D..M..... .......... .......... ...QE..... .TEDE.... .......... .......... ..........
Pleurodeles   ....L..... .S..E..V.. ....S..A ...M..G. ...ELQE... .NE...... .......... ..........
Odontesthes   ....H.D... .S..E..... ....SD.HL LG.~~..Y.. .GE.E..... ...D...... .......... .........S.
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Fig. 1. Amino-acid sequence of *DMRT1* (**a**) and *SOX9* (**b**) cDNA fragments in selected vertebrates. Underlined residues correspond to the 3′ half of the DM domain of *DMRT1* [Raymond et al., 1999; Kettlewell et al., 2000], and the 5′ portion of the HMG box of *SOX9* [Sudbeck et al., 1996]. Italic bold residues represent changes of polarity or charge among species that may be functionally important. Taxa labels (with *DMRT1* and *SOX9* accession numbers, respectively) are as follows: *Chrysemys = Chrysemys picta* (FJ791118; FJ804411), *Apalone = Apalone mutica* (FJ791119; FJ804412), *Trachemys = Trachemys scripta* (AY316537; EU914820), *Lepidochelys = Lepidochelys olivacea* (AF335421)*, Pelodiscus = Pelodiscus sinensis* (AB179697), *Crocodylus = Crocodylus palustris* (EU526604), *Mauremys = Mauremys reevesii* (AB365876), *Alligator = Alligator mississippiensis* (AF192560; AF106572), *Trimeresurus = Trimeresurus flavoviridis* (AB254801), *Elaphe = Elaphe quadrivirgata* (AB179698), *Gekko = Gekko hokouensis* (AB326222), *Calotes = Calotes versicolor* (AF464141; AF061784), *Gallus = Gallus gallus* (AF123456; U12533), *Homo = Homo sapiens* (BC040847; Z46629), *Rattus = Rattus norvegicus* (NM_053706), *Mus = Mus musculus* (AF202778; AF421878), *Xenopus = Xenopus laevis* (AB201112; AY035397), *Eublepharis = Eublepharis macularius* (AF217252), *Anas = Anas platyrhynchos* (AY904046), *Pleurodeles = Pleurodeles waltl* (EU872027), *Odontesthes = Odontesthes bonariensis* (AY319415).

ual upregulation at male-producing temperatures over stages 15–23, whereas expression at female-producing temperatures was consistently low during development [Kettlewell et al., 2000; Shoemaker et al., 2007a]. Although *DMRT1* expression in *L. olivacea* sea turtles is higher in males than in females, the expression trajectories differed from those in *T. scripta* as expression was consistently higher at male-producing temperature and showed a gradual downregulation over development at female-producing temperatures [Torres Maldonado et

Fig. 2. Developmental expression of *SOX9* and *DMRT1* in *Chrysemys picta* (TSD; panels **a** and **b**, respectively) and *Apalone mutica* turtles (GSD; panels **c** and **d**, respectively). The y-axis represents initial copy number normalized to β-actin (+ SD). Boxed stages in **a** and **b** correspond to the thermosensitive period of *C. picta*. Sample sizes at 25°C (bold) and at 30°C (italics) are presented per sampling time.

al., 2002]. Because AKGs were used in the present study, there is a possibility that subtle but otherwise biologically significant differences in gonadal expression may exist between temperatures that are masked by the AK (adrenal-kidney) expression [Pieau and Dorizzi, 2004; Ramsey and Crews, 2007; Shoemaker et al., 2007b]. On the other hand, when significant differences by temperature were detected using the AKG in those studies, they reflected differences in gonadal expression [Ramsey and Crews, 2007; Shoemaker et al., 2007b]. Thus, when AKGs are used, negative results (i.e., lack of differential expression) are more difficult to interpret, whereas when positive results are identified (i.e., differential expression), the observed patterns are likely a conservative estimate and can be attributed to gonadal differences. Importantly, since AKGs were also used by Kettlewell et al. [2000], the contrasting expression patterns between *C. picta* and *T. scripta* cannot be explained entirely by differences in the tis-

sues used between studies and may be biologically significant. Differences may also be attributed in part to the variation in expression among biological replicates used in this study compared to replicates obtained when samples from multiple individuals are pooled, and which may lead to statistical significance by reducing true biological variance that may exist. Nonetheless, research is ongoing to test this prediction directly by profiling gonadal and AK expression separately in painted and soft-shell turtles.

Similarly, *SOX9* expression in *C. picta* (fig. 2a) differed from that reported from other TSD turtles. In *T. scripta*, *SOX9* is upregulated from stage 17 at male-producing (low) temperatures [Shoemaker et al., 2007a], whereas in *L. olivacea* it is high for both sexes but is downregulated at female-producing (high) temperatures [Torres Maldonado et al., 2002]. However, it should be noted that for stages 15–22 in *C. picta*, *SOX9* profiles crisscrossed and

expression was upregulated at male-producing temperatures at stages 19 and 22 (albeit not significantly), similarly to what is observed in *T. scripta* [Shoemaker et al., 2007a]. In alligators, *SOX9* expression is basal in both sexes, but it is upregulated later in development at male-producing (high) temperatures [Western et al., 1999b], resembling *T. scripta*'s sex-specific expression pattern more closely than *Lepidochelys*'. Notably, in leopard geckos *SOX9* is highly expressed in both sexes until stage 36 after which it is downregulated at female-producing (low) temperatures [Valleley et al., 2001]. Expression profiles also crisscross in the leopard gecko at the onset of the thermosensitive period, consistent with observations in turtles [Shoemaker et al., 2007a; this study]. Therefore, in TSD reptiles *SOX9* expression appears to be consistently higher in males than in females at later stages of development via either its upregulation in males or its downregulation in females. Interestingly, this higher expression of *SOX9* in males at advanced stages of development is accomplished irrespective of the temperature that produces males in different TSD reptiles (lower temperatures in turtles but higher temperatures in alligators and leopard geckos). These observations reveal evolutionary changes in the regulation of common genes that form the network underlying sexual development in TSD taxa.

Importantly, *A. mutica* can be viewed as a negative control for thermal effects in *C. picta* [Valenzuela et al., 2006], because it is a GSD species whose gene expression is expected to be insensitive to temperature since it constitutes the average expression of presumably male and female embryos. Thus, finding a general effect of temperature on gene expression over most or all genes in *A. mutica* would suggest that gene expression is affected by temperature for reasons other than sex determination since temperature does not bias sex ratios in this species [Janzen, 1993]. Both *SOX9* and *DMRT1* expression was not affected by temperature in this taxon (fig. 2c, d), consistent with the findings for *SF-1* [Valenzuela et al., 2006], *aromatase* [Valenzuela and Shikano, 2007], and β-actin [Valenzuela et al., 2006] but in contrast to observations for *WT1* [Valenzuela, 2008b], and *DAX1* [Valenzuela, 2008a]. The thermal sensitivity of only 2 out of 7 genes in *A. mutica* rules out the existence of a general effect of temperature on the kinetics of gene expression, and instead lends support to the hypothesis that *A. mutica* harbors relic thermal sensitivity from its TSD ancestor [Valenzuela, 2008b]. Because we lack a molecular marker to identify the sex of *Apalone* embryos, the sex-specific patterns of gene expression remain an open question in this species that warrants further research.
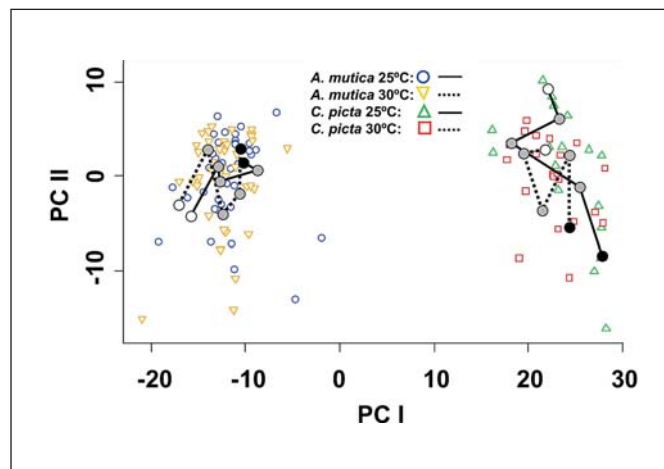
Fig. 3. Principal component plot of multivariate gene expression trajectories from *Chrysemys picta* and *Apalone mutica*. For all gene expression trajectories least square means at each developmental stage are shown; white circles for stage 9, gray for stages 12 to 19, and black for stage 22. Values from individual embryos are denoted by color symbols.

Multivariate Analysis of Gene Expression in GSD and TSD turtles

Results from the non-parametric MANOVA revealed a significant effect of developmental stage on the combined gene expression ($p < 0.0001$) and a temperature by stage interaction ($p = 0.0386$) in *C. picta*, indicating that overall, temperature had a significant effect on gene expression in this species, but the effect changed during embryonic development. In contrast, only the developmental stage had a significant effect on the combined gene expression in *A. mutica* ($p < 0.0001$). Consistently, PTA detected a significant difference in the orientation of the gene expression trajectories in multivariate space in *C. picta* at 25 and 30°C ($p = 0.019$; fig. 3), indicating that the relative covariation in gene expression varied between temperatures. Additionally, PTA also detected significant differences between species in the magnitude of gene expression change in the 25°C trajectories during development ($p = 0.0497$), and a marginally significant difference in the shape (i.e., in the pattern of covariation in gene expression) of the 30°C trajectories ($p = 0.0517$). These results indicate that the gene network underlying urogenital development responds differently to temperature in these 2 species with contrasting sex-determining mechanisms. Although *A. mutica* (GSD) exhibits differential expression by temperature in *WT1* [Valenzuela, 2008b] and *DAX1* [Valenzuela, 2008a], all 6 genes covary
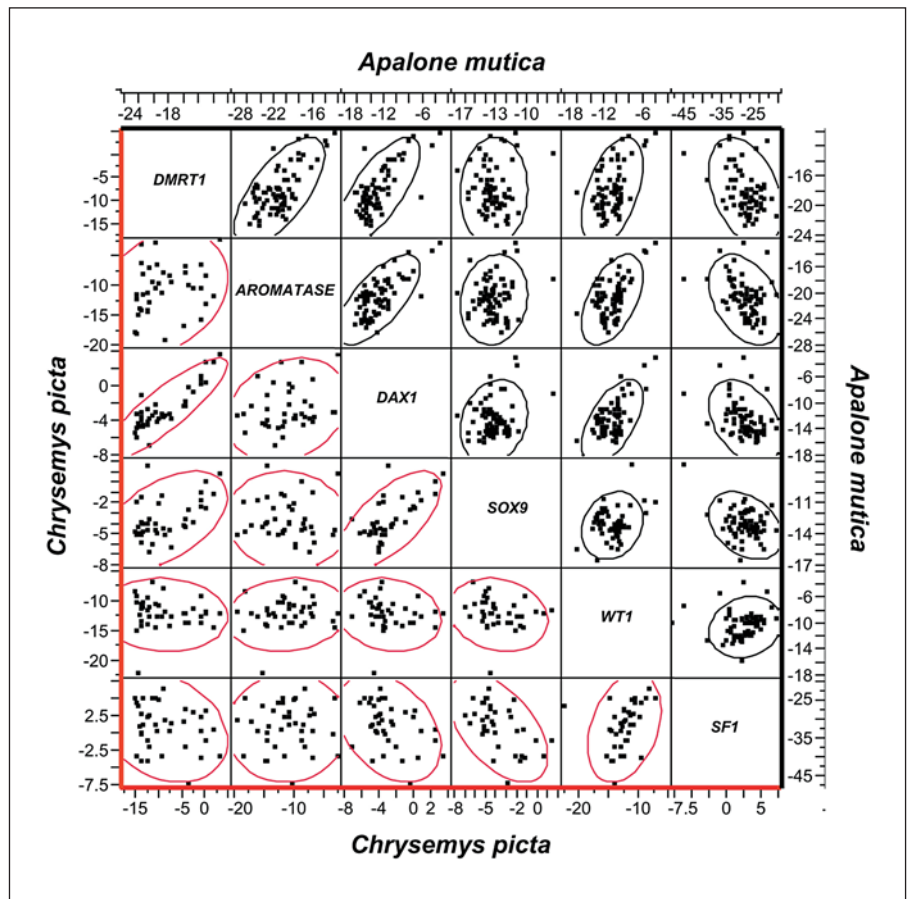
Fig. 4. Scatterplot matrix and 95% density ellipses of pairwise gene expression values for *Chrysemys picta* (red axes and ellipses) and *Apalone mutica* (black axes and ellipses).

in their expression in a thermo-insensitive manner. In contrast, in *C. picta* (TSD), the expression of *SF-1* [Valenzuela et al., 2006] and *WT1* [Valenzuela, 2008b] is sensitive to temperature as is also the covariation of all 6 genes. Given that when significant differences in gene expression by temperature are detected using the AKG they are likely due to differences in gonadal expression [Shoemaker et al., 2007b], I propose that this thermosensitive covariation in *C. picta* reflects a response of the gene network underlying gonadal development. Further research is warranted using AK and gonadal tissue separately to uncover additional differences from those detected here.

Analysis of the pairwise correlation of expression among genes detected a stronger covariation in gene expression in *A. mutica* than in *C. picta* (fig. 4), which would be expected for a developmental system that is more canalized, as may be the case if GSD evolved in the *A. mutica* lineage under directional selection from its TSD ancestor [Janzen and Krenz, 2004; Valenzuela,

2008b]. Similarly, partial correlation analysis, which tested for covariation between pairs of genes while taking into account their covariation with other genes in the network, revealed a more interconnected network in *A. mutica* than in *C. picta*, and a relatively more linear series of connections in the latter (fig. 5). Further research is needed to test whether such differences reflect a biologically significant effect of temperature in disassociating elements of the gene network underlying urogenital development in *C. picta* compared to *A. mutica* and thus, perhaps a fundamental difference between TSD and GSD systems. This network analysis also confirmed the functional relationships between genes hypothesized for these 2 turtle species [Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a, b], and for additional pairs of genes consistent with their role in male and female gonadal development in other TSD and GSD vertebrates [reviewed in Place and Lance, 2004; Valenzuela, 2008a].
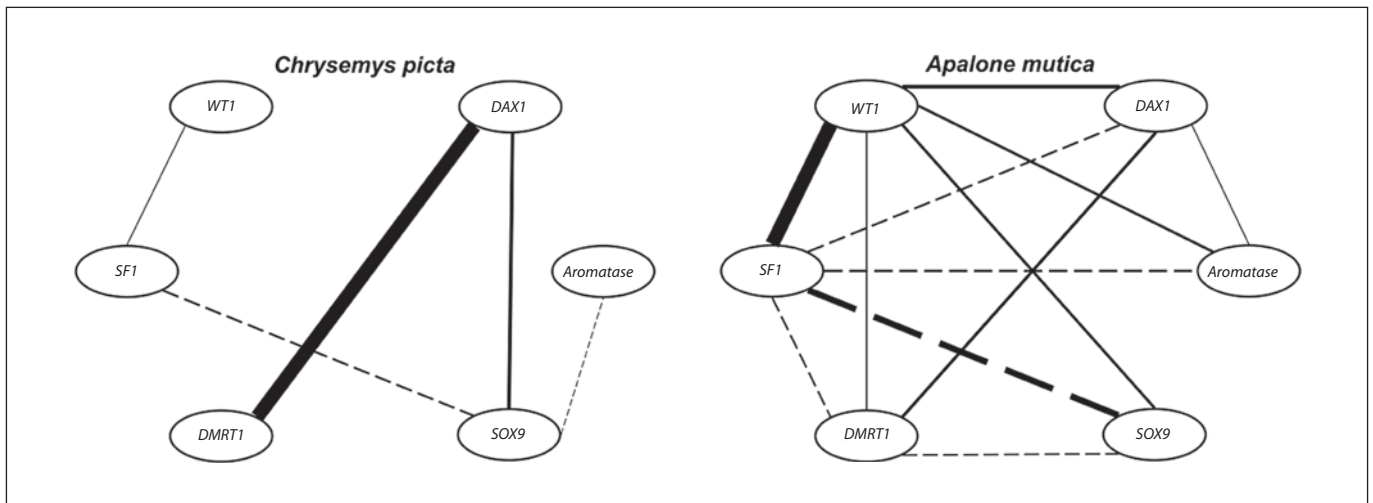
Fig. 5. Developmental gene coexpression network underlying urogenital development in *Chrysemys picta* and *Apalone mutica* using first-order conditional independence based on partial correlation. Significant partial correlations between gene pairs are indicated by solid (positive) and dashed (negative) lines, the width of which is proportional to the magnitude of the partial correlation within (not between) species. See text for details.

Conclusions

The network of genes and molecules underlying gonadogenesis remains incompletely characterized, even for mammals, both in terms of its composition and function. For instance, the lack of genes known to be expressed in the early mammalian ovary has precluded attempts to put together a pathway of gene regulation of early ovarian development [Wilhelm et al., 2007]. Thus, while relying on model systems to understand sexual development in reptiles has been a very fruitful endeavor, efforts to discover new elements of this network and their role in sex determination in reptiles can also shed important light on the regulation and evolution of this network in non-reptilian vertebrates. In particular, it is important to gain a comparative understanding of the composition and regulation of this network in closely related taxa with contrasting sex-determining mechanisms, as occurs in turtles and other reptiles.

The comparison of *DMRT1* and *SOX9* expression among vertebrates presented here confirms that significant differences in the expression trajectories exist among TSD turtles and other TSD vertebrates as has been reported for other genes involved in gonadal development [e.g. Valenzuela and Shikano, 2007; Valenzuela, 2008a; Merchant-Larios et al., 2009, this issue]. Future research to disentangle gonadal from AK expression will be able to uncover additional differences in gene expression from

those reported here and previously in these turtles [e.g. Valenzuela et al., 2006; Valenzuela, 2008b] and which are likely attributable to gonadal expression [Shoemaker et al., 2007b]. These observations reinforce the notion that the regulation of the gene network underlying sexual development has diverged over evolutionary time in vertebrates such that TSD is not a single trait from a developmental perspective [Valenzuela, 2008a]. Instead, the evidence indicates that TSD in vertebrates is another case of a polyphenism in which divergent network regulation is employed to produce conserved phenotypic outcomes, as occurs in other biological systems such as the wing polyphenism in ants [Abouheif and Wray, 2002].

The multivariate analysis of gene expression provided a more comprehensive view of the regulation of the network underlying sexual development in GSD and TSD turtles. This approach complements the information gained from the analysis of changes in the expression of individual genes over developmental time as it reveals emerging patterns that are undetectable by the single-gene approach. Here a testable hypothetical model for this gene network in *C. picta* and *A. mutica* was built based on partial correlations of gene expression, and should serve as the basis for further functional and evolutionary studies. Encouragingly, increasing multigene studies in reptiles [e.g. Crews et al., 2006; Rhen et al., 2007; Shoemaker et al., 2007a, b] should provide species-specific models of this gene network for comparative analysis.

The use of PTA to gene expression data presented here is another novel approach that allows exploring the co-variation in expression among genes, an important inherent component of developmental networks that is usually disregarded [see Rifkin and Kim, 2002]. The application of PTA to larger and other gene expression datasets in these and other taxa alone should provide important insights into evolutionary developmental biology and the emerging field of ecological genomics.

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