

Evolution of the gene network underlying gonadogenesis in turtles with temperature-dependent and genotypic sex determination

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Synopsis The evolution of sex determination has long fascinated biologists, as it has paramount consequences for the evolution of a multitude of traits, from sex allocation to speciation and extinction. Explaining the diversity of sex-determining systems found in vertebrates (genotypic or GSD and temperature-dependent or TSD) requires a comprehensive and integrative examination from both a functional and an evolutionary perspective. Particularly revealing is the examination of the gene network that regulates gonadogenesis. Here, I review some advances in this field and propose some additional hypotheses about the composition of the gene network underlying sexual development, the functional links among some of its elements and their evolution in turtles. I focus on several pending questions about: (1) What renders TSD systems thermo-sensitive? (2) Is there one developmentally conserved or multiple TSD mechanisms? (3) Have evolutionarily derived GSD species lost all ancestral thermal-sensitivity? New data are presented on embryonic expression of *Dax1* (the dosage-sensitive sex-reversal adrenal hypoplasia congenital on the X chromosome gene in the turtles *Chrysemys picta* (TSD) and *Apalone mutica* (GSD)). No differential *Dax1* expression was detected in *C. picta* at any of the stages examined, consistent with reports on two other TSD turtles and alligators. Notably, significantly higher *Dax1* expression was found at 30°C than at 25°C at stage 15 in *A. mutica* (GSD), likely caused by *Wt1*'s identical expression pattern previously reported. Because *Sfl* is an immediate downstream target of *Dax1* and its expression is not affected by temperature, it is proposed that *Sfl* renders *Dax1*'s differential signal ineffective to induce biased sex ratios in *A. mutica*, as previously proposed for *Wt1*'s thermosensitive expression. Thus, it is hypothesized that *Sfl* plays a major role in the lack of response of sex ratio to temperature of *A. mutica*, and may function as a sex-determining gene in this GSD species. These and previous data permit formulating several mechanistic hypotheses: (1) the postulation of *Wt1* as a candidate thermal master switch alone, or in combination with *Sfl*, in the TSD turtle *C. picta*; (2) the proposition of *Sfl* as a sex-determining gene in the GSD turtle *A. mutica*; and (3) the hypothesis that differing patterns of gene expression among TSD taxa reflect multiple traits from a developmental perspective. Moreover, the recent finding of relic differential *Wt1* expression in *A. mutica* and the results for *Dax1* in this species provide empirical evidence that GSD taxa can harbor thermal sensitivity at the level of gene expression, potentially co-optable during TSD evolution.

Introduction

The evolutionary survival of most dioecious organisms depends on the production of males and females sufficient to enable sexual reproduction. Surprisingly, the strong selection assumed to be acting to ensure that adults of both sexes are produced in adequate numbers has not resulted in a conserved mechanism of sex determination, but instead, it has generated an amazing array of sex-determining systems among taxa (Bull 1983; Solari 1994; Valenzuela et al. 2003; Valenzuela and Lance 2004). Explaining this diversity requires a comprehensive and integrative examination from both a functional and an evolutionary perspective. Particularly revealing in this quest is the

examination of the gene network that regulates gonadogenesis. Indeed, we must understand *how* alternative sex-determining mechanisms operate from an ecological and developmental point of view if we are to decipher *why* such recurrent transitions among mechanisms have taken place.

Sex determination is the process of irreversible commitment to the male or female developmental fate, which can be initially triggered by genetic factors such as those contained in sex chromosomes (GSD), or by environmental factors (ESD) such as temperature (TSD) (Bull 1983; Valenzuela and Lance 2004). Studies of model organisms have generated a wealth of information about the control of the

From the symposium "Reptile Genomics and Evolutionary Genetics" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 2–6, 2008, at San Antonio, Texas.

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Integrative and Comparative Biology, volume 48, number 4, pp. 476–485

doi:10.1093/icb/icn031

Advance Access publication May 13, 2008

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developmental steps of cell determination and cell differentiation that transform a unicellular zygote into an adult male or female (Gilbert 2000; Carroll et al. 2005). This has revealed the existence of a delicate balance between short-range and long-range signaling molecules that are required for the formation of the bipotential gonadal primordia, the determination to a male developmental fate or a female one, and the ensuing differentiation into testes or ovaries (Kim and Capel 2006). Comparative analyses have demonstrated that the gene regulatory network underlying these events contains numerous elements common to all vertebrates (reviewed in Place and Lance 2004) but is triggered by different initial activators in GSD and TSD systems.

Importantly, this gene network has been extensively studied in model organisms (Fig. 1), and only

more recently in non-model vertebrates, such as reptiles. Consequently, our current working paradigm for reptiles is built mostly upon fragmentary data from individual genes and species under the mostly untested assumption that the functional links of this network are largely the same as those known from model vertebrates. However, given that significant evolutionary divergence appears to have accrued in the elements of the gene network of sexual development (Wilkins 2002; Haag and Doty 2005; Valenzuela and Shikano 2007; Valenzuela 2008a), relying on a chimerical model may not reflect the true nature of this network and instead, it may obscure our functional and evolutionary understanding of it. For instance, if we are to decipher the nature of the evolutionary divergence of the gene network underlying sexual development to test

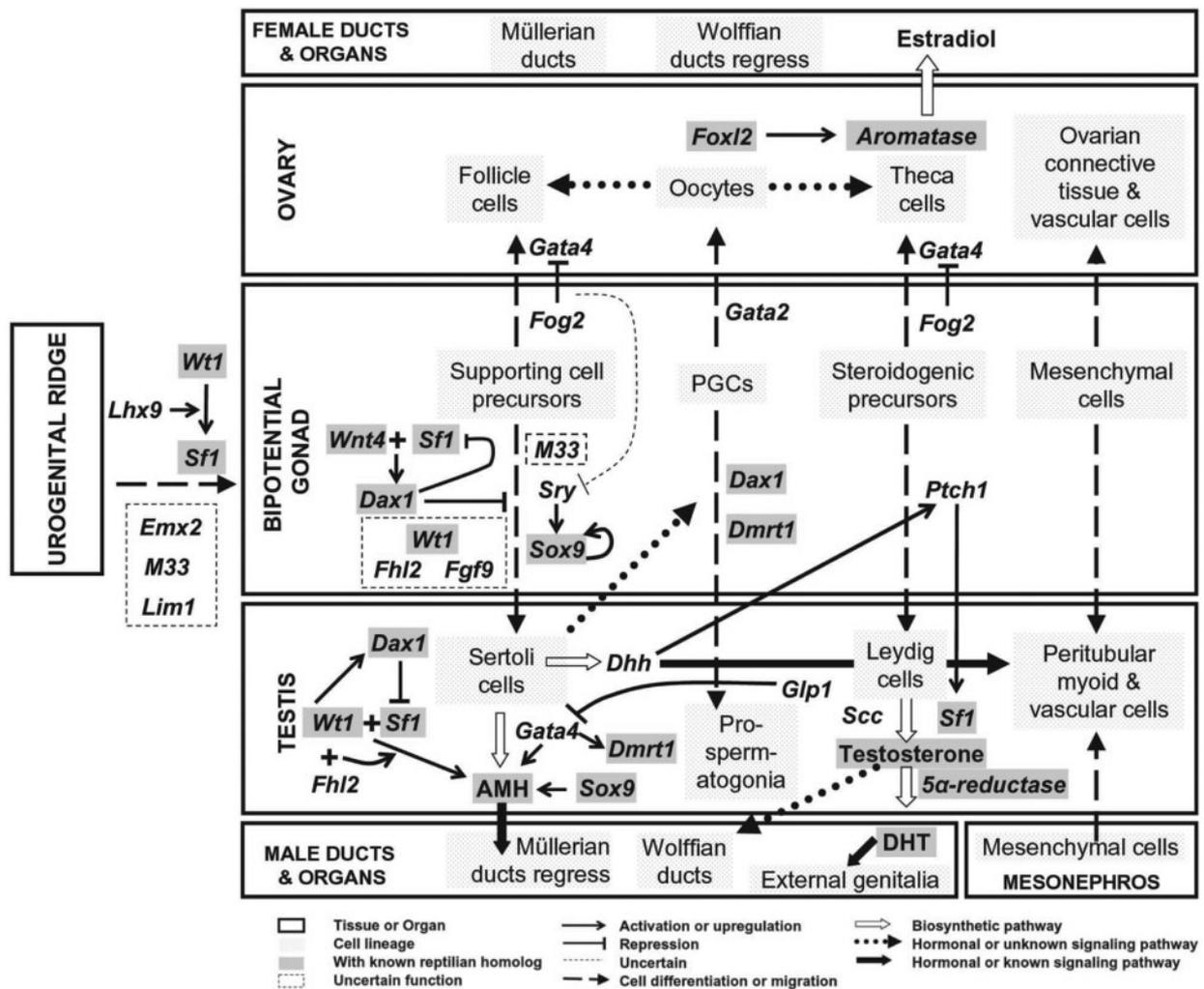


Fig. 1 Gene network and cellular interactions underlying gonadogenesis in GSD mammalian model systems, based on Koopman 2001 with modifications according to Lovell-Badge et al. 2002; Wilkins 2002; Yao et al. 2002; Lasala et al. 2004; Place and Lance 2004; Salmon et al. 2005; Crews et al. 2006; Li et al. 2007; Yamaguchi et al. 2007; Wilhelm et al. 2007. Elements with known reptilian homologs are indicated in the dark gray boxes. The list of elements of the gene network is not exhaustive. Key to symbols is included at the bottom of the graph.

current hypotheses about the mode in which such evolution occurred (e.g., Wilkins 1995, Graham et al. 2003, Nuzhdin et al. 2004), and the forces responsible for such changes (e.g., True and Haag 2001; Haag and Doty 2005), it is essential that we compare species-specific networks in non-model species. This is particularly crucial when studying evolutionary changes among closely related taxa that exhibit a wide variation of sex-determining mechanisms, as is the case of reptiles.

Reptilian homologs of mammalian and avian genes involved in gonadal differentiation have been identified (Fig. 1 gray boxes; e.g., *Aromatase*, *Amh*, *Dax1*, *Dmrt1*, *Foxl2*, *Sox9*, *Sfl*, *Wnt4*, *Wt1*), and indicate that common elements exist between GSD and TSD regulatory networks. Some of these genes exhibit temperature-specific expression in TSD species, concordant to their expression by sex in GSD taxa. Here I focus on four genes in particular: *Wt1*, *Sfl*, *Aromatase*, and *Dax1*.

Wt1, the Wilms' tumor-suppressor gene, encodes a transcription factor important for urogenital development (Kent et al. 1995; Roberts 2005) and it is necessary for the establishment of the bipotential gonad and later for the maintenance of Sertoli cells and seminiferous tubules in the testes of mammals (Gao et al. 2006, Fig. 1) and other vertebrates (e.g., Kent et al. 1995; Semba et al. 1996; Yamamura et al. 2005). *Sfl*, the steroidogenic factor 1 (or AD4BP and NR5A1) is a nuclear orphan receptor important for sexual differentiation in vertebrates, as it is required for the formation of primary steroidogenic organs (adrenal gland and gonad) in mammals, and for the expression of steroidogenic enzymes therein (Parker and Schimmer 1997; Morohashi 1999). *Aromatase* encodes the enzyme that converts androgens to estrogens and is important in many estrogen-dependent functions, including sexual differentiation (reviewed by Place and Lance 2004; Fig. 1). *Dax1* (dosage-sensitive sex-reversal [DSS], adrenal hypoplasia congenital [AHC] critical region on the X chromosome gene 1) encodes an orphan receptor that is a member of the nuclear receptor superfamily (McCabe 2007), and is involved in ovarian differentiation in mammals (Ramkissoon and Goodfellow 1996) and other vertebrates, including reptiles (Pieau et al. 1999; Western et al. 2000).

Important insights can be gained not only by comparing multiple elements of the gene network underlying sexual development, but also by contrasting patterns of gene expression across GSD and TSD reptiles from a network perspective. For instance, previous studies described significant differential expression of *Sfl* and *Wt1* at stage 12 in

Chrysemys picta turtles (TSD) prior to the onset of the thermosensitive period (TSP) (Valenzuela et al. 2006; Valenzuela 2008a). Additionally, *Wt1* expression was also affected by temperature at this early developmental stage in *Apalone mutica* turtles (GSD) (Valenzuela 2008a). Given that *Dax1* is a negative regulator of the transactivating functions of *Sfl*, particularly those mediated by *Wt1* (Iyer and McCabe 2004), and given that *Wt1* is also a known up regulator of *Dax1* (Fig. 1; Kim et al. 1999), it is important to examine the expression of *Dax1* in the context of what we know about other genes involved in gonadogenesis in these two taxa.

Dax1 has a C-terminal portion whose structure is characteristic of a ligand-binding domain, and an N-terminal portion formed by an atypical DNA-binding domain with 3.5 amino acid repeats (McCabe 2007). Importantly, *Dax1* has an inhibitory effect on *Sfl*-mediated transactivation of other genes. For instance, *Dax1* obstructs the combined synergism between *Wt1* and *Sfl* that is required during sexual development (Fig. 1) by the formation of heterodimers. Two *Dax1* spliceoforms exist at equal proportion in testis (Ho et al. 2004, Hossain et al. 2004). The most recently described alternative spliceoform is encoded by exon 1 and exon 2A, the latter of which is contained within the intron, between exons 1 and 2 (Ho et al. 2004; Hossain et al. 2004). *Dax1* exhibits sexually monomorphic expression in *Trachemys scripta*, *Lepidochelys olivacea*, and *Alligator mississippiensis* (Western et al. 2000; Maldonado et al. 2002; Shoemaker et al. 2007b).

Here, I analyze *Dax1* expression during the embryogenesis of *C. picta* and *A. mutica*, and formalize some hypotheses about the composition of the gene network underlying sexual development, the functional links among some of its elements and their evolution in turtles, based on these novel and previous data.

Materials and methods

Collection of samples

Freshly laid eggs were collected from natural nests of *C. picta* (7 clutches) and *A. mutica* (17 clutches), and correspond to the same eggs used previously (Valenzuela et al. 2006). Eggs were incubated at constant temperatures that produce ~100% male (25°C) and ~100% female (30°C) *C. picta* hatchlings (Ewert and Nelson 1991). Temperature does not bias sex ratios in *A. mutica* (GSD) (Janzen 1993).

Eggs from each clutch were uniformly distributed between incubation temperatures. Eggs from each clutch were divided equally among multiple plastic

Table 1 Primers used for standard PCR or quantitative-real time PCR (those with *qm* prefix) amplification of *Wt1* and β -actin cDNA fragments in *Chrysemys picta* and *Apalone mutica*

Primers	<i>Chrysemys picta</i>	<i>Apalone mutica</i>
β -actin forward	5'-CAGGTCATCACCATYGGCAA-3'	
β -actin reverse	5'-GCTTGCTGATCCACATCTGC-3'	
<i>Dax1</i> forward	5'-GGAGTACGCCTACCTCAAGGG-3'	
<i>Dax1</i> reverse	5'-CATYTCCAGCAKCATRTCATCC-3'	
<i>qm</i> β -actin forward	5'-AAGCCCTCTTCCAGCCAT-3'	5'-AAGCTCTTCCAGCCCT-3'
<i>qm</i> β -actin reverse	5'-GACAGCACAGTGTGGCG-3'	
<i>qmDax1</i> forward	5'-CAGGCCAGATTTGCCAAG-3'	5'-CCAGGGCTGCAGTGTGTACA-3'
<i>qmDax1</i> reverse	5'-TGTTCCAATGATGGGCCT-3'	5'-TCAGTGTGATGTGTTCAATTTAGACTT-3'

Blank cells indicate that the same primers were used for both species because sequences were identical.

containers filled 3/5 of the way with moistened vermiculite set at -150 kPa by adding 338 g of water per 300 g of vermiculite. Boxes containing only the substrate were weighed (± 0.1 g), and they were weighed again after adding the eggs and the weight recorded. Eggs were partially buried in the substrate in random positions in the box. Prior to the sampling of any egg (or weekly otherwise), the boxes were reweighed and lost moisture (as determined by weight loss) was replaced. The new weight was recorded after the removal of any eggs. Egg containers were rotated at least weekly to control for the effect of any potential thermal cline in the incubators.

Embryos were collected at several developmental stages prior to and during the TSP (*sensu* Yntema 1968) equivalent for both temperature treatments and species used: Stage 9 = Before TSP, Stage 12 = Before TSP, Stage 15 = TSP Onset, Stage 19 = Middle TSP, Stage 22 = End TSP (Bull and Vogt 1981). Collection of embryos from each clutch was also distributed across all developmental stages sampled. Sampled embryos were placed immediately in 10 vol. of RNAlater[®], stored at -20°C and subsequently at -80°C for later use, following the manufacturer's recommendations.

Cloning and QPCR

RNA was extracted from the adrenal-kidney-gonadal complex (whole embryos or trunks were used from stages 9 and 12 embryos, respectively) using QIAGEN's RNeasy Kits and DNase-I digested to prevent DNA contamination. 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 $\mu\text{g}/\text{sample}$) was retro-transcribed with (dT)₂₀ primers using Superscript III

(Invitrogen) following the manufacturer's protocol. For samples that yielded <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 for relatively conserved regions of vertebrate *Dax1* cDNA sequences as found in GeneBank (Table 1). These primers amplified a 268 bp *Dax1* cDNA fragment in both species, which was cloned in a pGEM[®]-T Easy Vector System (Promega) and sequenced. A 343 bp β -actin cDNA fragment (Valenzuela et al. 2006) was used to test for the quality of the extracted RNA during QPCR, and for normalization of *Dax1* gene expression. Internal primers amplified a 124 bp β -actin fragment (Valenzuela et al. 2006), and 98 and 79 bp *Dax1* fragments in *C. picta* and *A. mutica*, respectively (Table 1), during real-time QPCR using Brilliant[®] SYBR[®] Green QPCR Master Mix in an Mx3000P thermocycler (Stratagene). ROX was used as the 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 followed Valenzuela et al. (2006): 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 obtained at the end of the PCR cycles were inspected to confirm amplification of a single transcript.

Analysis of data

Initial template amount for each sample and each gene was calculated using standard-curve quantification via the algorithms implemented in 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 μ g of initial total RNA. Expression of *Wt1* was normalized using β -*actin* expression data, and Log_2 transformed to correct for the heteroscedasticity and nonnormal distribution characteristic of amplification data. Statistical analysis was carried out on these normalized Log_2 -transformed data keeping individual values separate (Valenzuela et al. 2006). The significance of the temperature-treatment effect was determined by testing for differences in the mean values of gene expression at each sampled developmental stage via an ANOVA as implemented in Jmp 5.1.2[©] (SAS Institute 2004).

Results

The fragments of *Dax1* cloned from embryonic adrenal-kidney-gonadal tissue encompassed part of the 3' end of the exon 1 and part of the 5' region of the exon 2, which correspond to the C-terminal ligand-binding domain and share high similarity with other members of the nuclear hormone-receptor-gene superfamily (Guo et al. 1996; McCabe 2007). Given the location of the primers, the transcript abundance measured by QPCR does not include the recently described alternative spliceoform (Ho et al. 2004; Hossain et al. 2004). The sequence of the *Dax1* fragment of *C. picta*'s (GenBank EU433884) was 93.6% identical to *A. mutica*'s (GenBank EU433885), 97.8% to *T. scripta*'s, 95.0% to *L. olivacea*, 91.8% to alligators's, 86.9% to chicken's, 77.0% to human's, 72.4% to those of mice. Differences were also found at the amino acid level (Table 2). The amino acid sequence of the *Dax1* fragment of *C. picta*'s was 93.3% identical to *A. mutica*'s, 95.6% to *T. scripta*'s, 94.1% to *L. olivacea*, 92.2% to alligators's, 91.1% to chicken's, 74.4% to human's, 71.1% to those of mice. The amino acid sequences of *C. picta* and *A. mutica* differ by three residues from that of *T. scripta* (Shoemaker et al. 2007) but the changes are unique to each of these two species (Table 2).

Standard curves for β -*actin* and *Dax1* had R^2 values of 0.97 and 0.998, respectively, for *C. picta*, and of 0.98 and 0.991, respectively, for *A. mutica*. No significant differences in *Dax1* expression were detected in *C. picta* at any of the stages examined. Notably, in *A. mutica*, *Dax1* expression was

significantly higher at 25°C than at 30°C during stage 15 ($F=5.2593$, $P=0.0323$; Fig. 2).

Significance of the results is neither due to any systematic bias generated by a small number of clutches represented, nor to clutch-mates being over-represented in any particular developmental stage or temperature. For *A. mutica*, 17 clutches were represented, and a single egg per clutch was sampled at all temperature-by-stage combinations. For *C. picta*, 7 clutches were represented, and only in stages 19 and 22 did two of the total eggs sampled at each temperature come from the same clutch. Readings from these clutch-mate pairs were not more similar than from any pair of unrelated individuals sampled at the same temperature-by-stage combination. Additionally, although duplicate readings from embryonic samples were precluded because the limited amount of total RNA per sample was used to profile seven different genes (Valenzuela et al. 2006; Valenzuela and Shikano 2007; Valenzuela 2008a and unpublished data), duplicate readings from the standard curves were 97–99.9% identical, thus ensuring the technical reliability of the readings of transcript abundance.

Discussion

An important step in explaining the outstanding lability of sex-determining mechanisms requires a better understanding of the regulation and evolution of the gene network that controls sexual development. Taxonomic groups such as reptiles that encompass a wide variety of mechanisms are particularly informative as they allow a comparative analysis of evolutionary changes that have accrued among closely related taxa with alternative sex-determining systems. Such is the case of the target species used in this study, *C. picta* (TSD) and *A. mutica* (GSD), which have been the subject of recent comparative analyses of developmental gene expression (Valenzuela et al. 2006; Valenzuela and Shikano 2007; Valenzuela 2008a).

In this study, expression of *Dax1* was insensitive to temperature at all stages examined. Consistently, *Dax1* expression during the TSP of developing gonads of *T. scripta*, *L. olivacea*, and of the gonad-adrenal-mesonephros of *A. mississippiensis* was not significantly different between male-producing and female-producing temperatures (Western et al. 2000; Maldonado et al. 2002; Shoemaker et al. 2007b). However, significant differential *Dax1* expression was detected at stage 15 of development in *A. mutica*. The potential functional and evolutionary significance of this observation emerges when this pattern of expression is compared to that of other genes of

Table 2 Amino-acid sequence of *Dax1* cDNA fragments in selected species of vertebrates

	10	20	30	40	50

<i>Trachemys</i>	KEYAYLKGTV	LFNPDLPGLQ	CVQYIQGLQR	EAQQALNEHV	<u>TLIHQ</u> GDQAR
<i>Lepidochelys</i>R.	<u>R.L.R</u>
<i>Chrysemys</i>	X.....I <u>R</u>
<i>Apalone</i>	X.....P.	<u>R</u>
<i>Alligator</i>	X.....A.T.....	R...R.....
<i>Gallus</i>	X.....T...E...K	...E.....	R...R.....
<i>Mus</i>	X.....K...E...W	RT...I.T..I	RMMQREY.I.
<i>Homo</i>V....	..K.....W	GT...I.S..T	RMT...PHD.
	60	70	80	90	
	
<i>Trachemys</i>	FAKLNVLVLSL	LRSINANVIA	ELFFRPIIGT	VNMD	DDMLLEM
<i>Lepidochelys</i>Q..	
<i>Chrysemys</i>M...
<i>Apalone</i> <u>T</u>
<i>Alligator</i>	IA...
<i>Gallus</i>S
<i>Mus</i>	S.E..SA.F.	..F..SD.VT	A .S....M...	
<i>Homo</i>	.IE..ST.F.	..F.....S....M...	

Trachemys, *T. scripta*; *Lepidochelys*, *L. olivacea*; *Chrysemys*, *C. picta*; *Apalone*, *A. mutica*; *Alligator*, *A. mississippiensis*; *Gallus*, *Gallus gallus*; *Mus*, *Mus musculus*; *Homo*, *Homo sapiens*. Underlined bold residues represent changes of polarity or charge among turtles that may be functionally important.

the sexual development network in a comparative framework.

For instance, results from previous studies on *C. picta* and *A. mutica* permit the examination of several important questions. One is the still pending question of what renders TSD systems thermo-sensitive. Such a thermal master switch will be a gene(s) that activates the TSP or that enables responses during this developmental window of time. Candidates for this role would be genes whose expression occurs prior to, or exactly at, the onset of the TSP, rather than genes exhibiting differential expression within the TSP after it has already been activated. Because early *Sfl* expression is required for the formation of bipotential gonads (Wilkins 2002, Fig. 1), *Sfl* was recently proposed as one such potential master switch in the TSD turtle *C. picta*, based on its early significant differential expression

at stage 12, prior to the onset of the TSP (Valenzuela et al. 2006). However, *Wt1* was also found to have significant differential expression at stage 12 in *C. picta* (Valenzuela 2008a). Early *Wt1* expression is also required for the formation of the bipotential gonads (Gao et al. 2006, Fig. 1). Because *Wt1* is known to regulate *Sfl* in other vertebrates (Wilhelm and Englert 2002), *Wt1* was proposed as another candidate master switch in TSD turtles (Valenzuela 2008a), which, given the regulatory function of *Wt1* on *Sfl*, makes it an even more likely candidate than *Sfl* (Fig. 3). The hypothetical role of *Wt1* (alone, or in combination with *Sfl*) as a thermal master switch is consistent with the evolutionary developmental model which proposes that the hierarchy of the genetic pathway underlying sex determination evolves from the bottom-up as a series of evolutionary steps that favor the addition of neomorphic

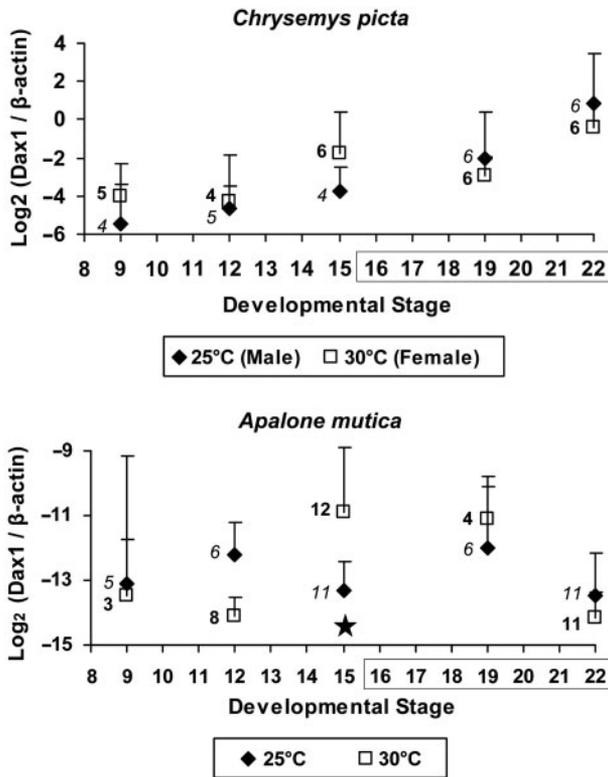


Fig. 2 Developmental expression of *Dax1* in *C. picta* (TSD) and *A. mutica* turtles (GSD). Y-axis represents *Dax1* initial copy number normalized to β -actin (+SD). Boxed stages correspond to *C. picta*'s TSP. Sample sizes at 25°C (italics) and at 30°C (bold) are presented per sampling time. Stars denote significant differential expression. Results are robust irrespective of outliers. Differences in transcript abundance at all other stages are not statistically significant and thus, no biological significance can be attributed to the crossing of the expression profiles.

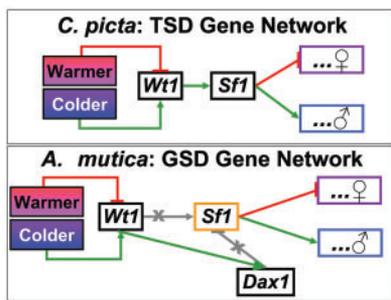


Fig. 3 Proposed hypothetical models of developmental gene networks regulating gonadogenesis under TSD and GSD. Models are based on patterns of gene expression from *C. picta* and *A. mutica* as described in the text. Warmer and colder refers to incubation temperature during development. Arrows indicate positive regulation or activation, and lines with end caps indicate negative regulation or repression. An X on a line indicates a signal that has been rendered ineffective. Dots within the male and female boxes denote all remaining gene and cellular interactions leading to sexual development as summarized in Fig. 1.

regulators upstream of the cascade (Wilkins 1995; Graham et al. 2003).

A second question is whether TSD encompasses a single or multiple mechanisms. This relates to the fundamental question of whether TSD mechanisms evolved in one developmentally conserved way, or whether multiple molecular pathways evolved to produce ecologically equivalent outcomes (e.g., different TSD molecular pathways may exist that result in the same sex-by-temperature reaction-norm, such as the pattern of male-to-female production with increasing temperature or TSDIa). The diversity of systems harbored within GSD (Wilkins 2002) provides evidence that such evolutionary changes have occurred in the evolution of sex-determining mechanisms, rendering the possibility for similar changes within TSD more likely. Alternatively, concordant expression of genes across species would be expected if TSD systems are pleiomorphic and developmentally conserved. A comparative analysis of data from *C. picta* and *A. mutica* with that of other turtles reported in the literature, indicated that substantial evolutionary changes have accrued not only between TSD and GSD systems, but among TSD species as well (Valenzuela et al. 2006; Valenzuela and Shikano 2007; Valenzuela 2008a) in genes such as *Sf1*, *Aromatase*, and *Wt1*. These differences at the level of gene expression between TSD species suggest that there are multiple TSD systems that differ in their molecular underpinnings of development (*sensu* Abouheif and Wray 2002), perhaps as the result of divergence over evolutionary time (True and Haag 2001; Nuzhdin et al. 2004). Importantly, this comparative analysis also revealed that the fundamental difference between TSD and GSD mechanisms is not driven by *Aromatase* as previously proposed (reviewed by Pieau and Dorizzi 2004) because patterns of either differential or insensitive aromatase gene expression or activity during the TSP are found across TSD and GSD vertebrates (Valenzuela and Shikano 2007).

A third important issue is whether gene expression is totally thermo-insensitive in GSD taxa, or not. For turtles, whose ancestral state appears to be TSD (Janzen and Krenz 2004), this relates to the evolutionary question of whether GSD turtles have lost all thermal sensitivity in the regulation of the gene network underlying sexual development. Data from *A. mutica* provided evidence that this is not always the case, because this GSD turtle has retained its ancestral sensitivity to the expression of a gene involved in gonadogenesis, *Wt1*, the first such case ever to be reported (Valenzuela 2008a). The finding of differential expression in *Wt1* in *A. mutica* is important because such a thermally sensitive gene

may have been the key factor responsible for the thermal sensitivity of the TSD ancestor. Consistent with this idea, *Wt1* was independently proposed as a potential thermal master switch based on gene-expression data from *C. picta* (Valenzuela 2008a). Of further relevance to the *Dax1* results obtained here, it should be pointed out that such relic thermal-sensitivity must be overridden now by the master sex-determining gene in the GSD *A. mutica*.

At stages 12 and 15 of development, *A. mutica* exhibited significant and marginally significant differential *Wt1* expression, respectively (Valenzuela 2008a). Remarkably, the expression pattern of *Dax1* at those two stages mimics the pattern of *Wt1* and the transcript abundance values of *Wt1* and *Dax1* are highly correlated with one another ($r=0.999$, $P=0.0013$). Because *Wt1* is a known upregulator of *Dax1* (Fig. 1; Kim et al. 1999), this result supports the functional link between *Wt1* and *Dax1* in *A. mutica*. The question follows as to why it is that the differential expression of *Dax1* at stage 15 does not produce biased sex ratios in this species? The data point once more to *Sfl* (Valenzuela 2008a). *Dax1* inhibits the function of *Sfl* in the transactivation of several other genes (Iyer and McCabe 2004). Because *Sfl* is a downstream target of *Dax1*, and because *Sfl* is insensitive to temperature in *A. mutica* (Valenzuela et al. 2006), *Dax1*'s thermal signal is rendered ineffective by *Sfl* in the same manner that *Wt1*'s signal appears to be (Valenzuela 2008a, Fig. 3). Thus, the two lines of evidence (this study and that of Valenzuela 2008a) indicate *Sfl* has an important role in controlling the lack of response to temperature of the sex ratio in *A. mutica*. I propose that *Sfl* may function as a sex-determining gene in this GSD species. Further genomic and functional research is necessary to test this hypothesis.

In summary, it is argued here that a more comprehensive understanding of the functional composition and evolution of the gene network underlying sexual development requires a comparative approach at the level of the genes and species. Such efforts using *C. picta* and *A. mutica* have enabled the formulation of a series of mechanistic hypotheses. These include (1) the postulation of *Wt1* as a candidate thermal master switch alone or in combination with *Sfl* in the TSD turtle *C. picta* (Fig. 3); (2) the proposition of *Sfl* as a sex-determining gene in the GSD turtle *A. mutica* (Fig. 3); and (3) the hypothesis that differing patterns of gene expression among TSD taxa reflect multiple traits from a developmental perspective. Moreover, the finding of relic differential *Wt1* expression in *A. mutica* and the results for *Dax1* in this same species provide empirical evidence that

GSD taxa can harbor thermal sensitivity at the level of expression of genes involved in gonadal formation. This thermosensitive gene expression in GSD taxa represents a co-optable source for the evolution of phenotypic plasticity such as TSD, as previously proposed (Bull 1981, 1983; reviewed in Valenzuela 2004).

More research is needed to elucidate the forces responsible for the evolutionary divergence of gene expression patterns. Encouragingly, research on reptiles using a multigene approach are increasing (e.g., Maldonado et al. 2002; Rhen et al. 2007; Shoemaker et al. 2007a, b) and that should help in generating species-specific models of this gene network. It should be noted that more attention should be paid to other aspects of the regulatory gene network underlying sexual development. For instance, heterochronic changes (Alberch et al. 1979) in the timing of specification and determination between TSD and GSD mechanisms will alter the function of this network in ways that may alleviate the strong selection on the master trigger of male and female sexual development, thus facilitating the evolutionary transitions between GSD and TSD (Valenzuela 2008b). An integrated approach that includes those considerations, along with research on the ecological context in which alternative gene expression patterns are found in nature, should help reveal how males and females are produced, and the forces responsible for generating the existing variety of sex-determining mechanisms.

Acknowledgments

I thank T. Shikano and A. LeClere for their assistance with some collection of data. This study was partially funded by the Center for Integrative Genomics at Iowa State University, and by NSF grant IOS 0743284 to Nicole Valenzuela.

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