

Transcriptional Evolution Underlying Vertebrate Sexual Development

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Explaining the diversity of vertebrate sex-determining mechanisms ranging from genotypic (GSD) to temperature-dependent (TSD) remains a developmental and evolutionary conundrum. Using a phylogenetic framework, we explore the transcriptional evolution during gonadogenesis of several genes involved in sexual development, combining novel data from *Chrysemys picta* turtles (TSD) and published data from other TSD and GSD vertebrates. Our novel *C. picta* dataset underscores *Sf1* and *Wt1* as potential activators of the thermosensitive period and uncovered the first evidence of *Dax1* involvement in male development in a TSD vertebrate. Contrasting transcriptional profiles revealed male-biased *Wt1* expression in fish while monomorphic expression is found in tetrapods but absent in turtles. *Sf1* expression appears highly labile with transitions among testicular, ovarian, and non-sex-specific gonadal formation patterns among and within lineages. *Dax1*'s dual role in ovarian and testicular formation is found in fish and mammals but is dosage-sensitive exclusively in eutherian mammals due to its X-linkage in this group. Contrastingly, *Sox9* male-biased and *Aromatase* female-biased expression appear ancestral and virtually conserved throughout vertebrates despite significant heterochronic changes in expression as other elements likely replaced their function in early gonadogenesis. Finally, research avenues are highlighted to further study the evolution of the regulatory network of sexual development. *Developmental Dynamics* 242:307–319, 2013. © 2012 Wiley Periodicals, Inc.

Key words: environmental and genotypic sex determination; regulatory gene network; embryonic development; evolutionary adaptation; natural selection; heritability; transcription mRNA; gene expression; vertebrates; birds; reptiles; mammals; amphibians; fish

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INTRODUCTION

Vertebrate primary sexual development is controlled by a gene network that regulates sex determination (the commitment to the male or female gonadal fate) and sex differentiation (the development of sex-specific cellular and other phenotypes) (Parma and Radi, 2012). Most elements of this gene regulatory network are common to all vertebrates (Morrish and Sinclair, 2002; Place and Lance, 2004;

Rhen and Schroeder, 2010). Yet, sex-determining mechanisms vary widely among lineages and range from genotypic to environmental (Fig. 1) (Bull, 1983; Valenzuela et al., 2003). These differences emerge in part by the recruitment of different master regulators of this regulatory network (Zarkower, 2001; Haag and Doty, 2005; Wilkins, 2005). Much theoretical and empirical work has been devoted to identifying these master triggers

(Koopman et al., 1990; Sinclair et al., 1990; Raymond et al., 1999; Matsuda et al., 2002; Yoshimoto et al., 2008), modeling their inheritance (Bull, 1983; Valenzuela et al., 2003; Sarre et al., 2011), and analyzing the causes and consequences of the evolutionary transitions in their emergence or loss (Shine, 1999; Valenzuela, 2004, 2008c 2010a; Uller and Badyaev, 2009; Bachtrog et al., 2011; Sarre et al., 2011). Here, we

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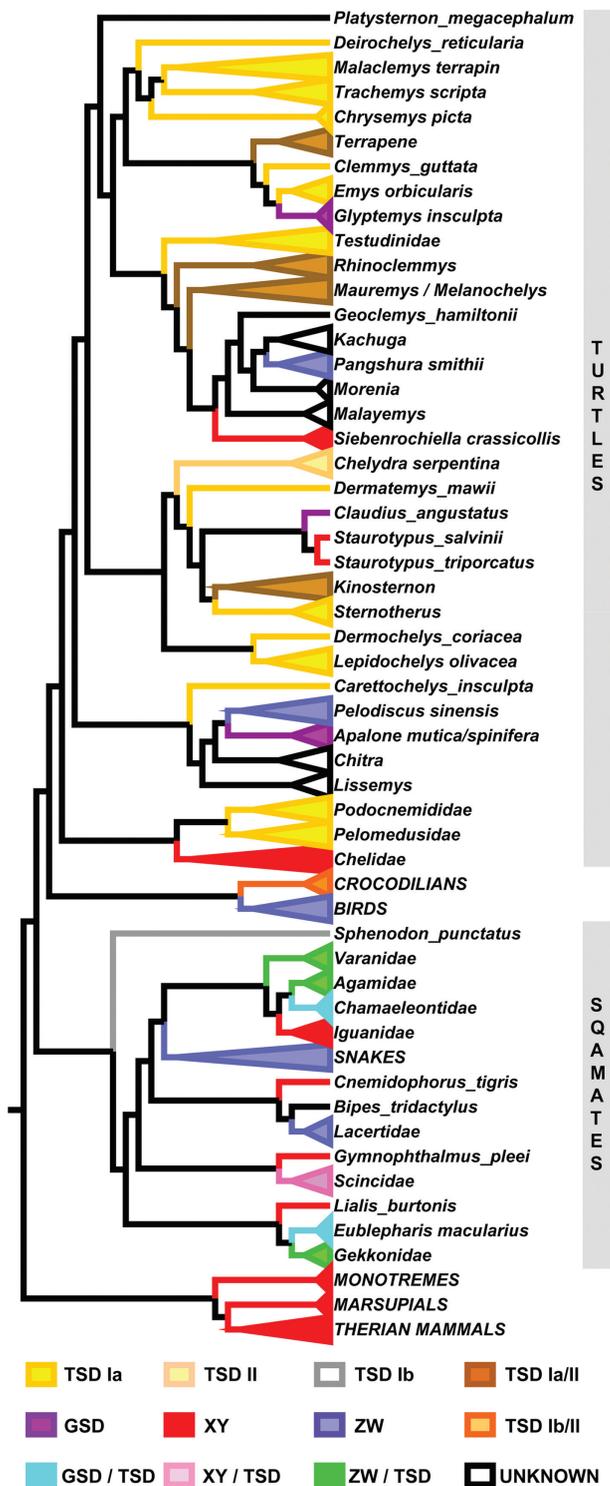


Fig. 1. Phylogenetic relationships among vertebrates with special emphasis on non-avian reptiles, and their diverse sex-determining mechanisms. Triangular branches denote collapsed taxonomic Orders/Suborders/Families, or multitypic lineages represented by the species listed as the tip label. In the latter case, the color indicates the sex-determining mechanisms of the exemplary species listed at the tip.

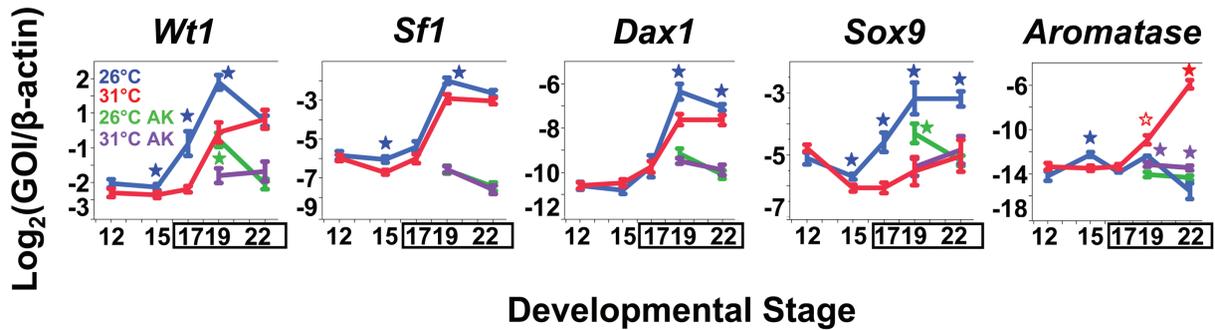
use a phylogenetic context to explore a less studied aspect that contributes to the diversity of sex-determining mechanisms, namely, the

rewiring or adjustment of co-expression patterns of gene modules in this regulatory network, downstream of the master triggers.

Approaches to understand the composition, regulation, and evolution of the regulatory network underlying sexual development include classical developmental biology methods using mutagenesis in model systems to uncover the effect of mutated genes compared to wild type phenotypes (Arboleda and Vilain, 2011), while studies in non-model organisms have relied mainly on finding orthologs of the genes originally identified in model systems and comparing the expression patterns (Valleley et al., 1992, 2001; Smith et al., 1995; Bergeron et al., 1998; Spotila et al., 1998; Western et al., 1999, 2000; Rhen and Crews, 2001; Torres-Maldonado et al., 2002; Murdock and Wibbels, 2003; Shoemaker et al., 2007b; Hoshi and Nakao, 2008; Capriglione et al., 2010; Valenzuela, 2010b). Leveraging more modern genomic technologies permits the de novo interrogation of transcriptomes in non-model systems to uncover species-specific candidate genes involved in sexual development (Munger et al., 2009; Hale et al., 2010). However, comparative transcriptomic studies across vertebrates that include non-model taxa are lacking.

Thus, here we concentrate on a small panel of genes (*Wt1*, *Sf1*, *Dax1*, *Sox9*, and *Aromatase*) that have been well characterized in amniotic vertebrates from several taxonomic orders across genotypic (GSD) and environmental sex determination (temperature-dependent sex determination or TSD), and review their expression during embryonic development of males and females across birds, reptiles, mammals, amphibians, and fish. Notably, we show that gene expression and co-expression patterns vary among vertebrates, which suggests evolutionary changes in gene regulatory networks. Several other genes have been studied in model and non-model vertebrates under different conditions (for a review see Place and Lance, 2004; Rhen and Schroeder, 2010; Parma and Radi, 2012).

Because intriguing patterns of gene expression at constant temperature were reported recently for *Chrysemys picta* turtles, which, due to some logistical limitations of those studies, warranted further investigation (Valenzuela et al., 2006; Valenzuela



Abbreviations:	Expression by Treatment and Tissue:	Significantly Higher Expression at $\alpha=0.05$:
MPT = male-producing temperature	Blue = trunks, AKG, or G at MPT	★ = in AKG or G at MPT
FPT = female-producing temperature	Green = AK at MPT	★ = in AK at MPT
NM = natural male profile	Red = trunks, AKG, or G at FPT	★ = in AKG or G at FPT
NF = natural female profile	Purple = AK at FPT	★ = in AK at FPT
GOI = gene of interest	Note that:	★ = not significant since $n = 1$ at 26°C
AKG = adrenal/kidney/gonad complex	•Boxed stages = thermosensitive period	
AK = adrenal/kidney	•Stages 19&22 have two separate data points corresponding to AK and G	
G = gonad		

Fig. 2. Transcriptional response (mean and standard errors) of *Wt1*, *Sf1*, *Dax1*, *Sox9*, and *Aromatase* in painted turtles (*Chrysemys picta*) incubated at constant male- and female-producing temperatures. Sample sizes as in Table 2.

and Shikano, 2007; Valenzuela, 2008a,b, 2010b), we present here novel data from constant incubation experiments to test the validity of previous observations and highlight the discovery of novel patterns and their evolutionary implications.

RESULTS AND DISCUSSION

Novel Gene Expression Data From *Chrysemys picta*

Our new extended dataset from constant temperatures revealed that *Wt1* and *Sf1* show differential expression prior to the onset of the thermosensitive period or TSP (Fig. 2). This result is consistent with previous findings in *C. picta* and supports the hypothesis that *Wt1* and *Sf1* may play a role as activators of the TSP and act as TSD master switches of sex-specific development (Valenzuela, 2008a). Differential expression was also detected for the first time in *Sox9* and *Aromatase* at this early developmental stage (Fig. 2) suggesting that they, too, might mediate the activation of the TSP along with *Sf1* and *Wt1*. This novel observation of early differential *Aromatase* expression in a TSD species is concordant with markedly early *Aromatase* expression reported in other vertebrates, and suggests that *Aromatase* has functions that are independ-

ent of ovarian formation early in development (Walters et al., 2000).

Importantly, by profiling the gonadal expression separated from the adrenal/kidney (AK) tissue during the TSP, we were able to detect significant differences between the male and female gonadal pathways that were obscured when using AKGs in the past in this and other species (Willingham et al., 2000; Pieau and Dorizzi, 2004; Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a,b, 2010b). For instance, *Dax1*, exhibited a male-biased gonadal expression during the TSP in our study, the first report of a significant temperature effect on *Dax1* expression in a TSD species. In contrast, previous studies on other TSD turtles and American alligator had not detected differences in expression between male- and female-producing temperatures (Western et al., 2000; Torres-Maldonado et al., 2002; Rhen et al., 2007; Shoemaker et al., 2007a). Likewise, our results show that during the TSP, gonadal expression of *Wt1*, *Sf1*, *Sox9*, and *Aromatase* in *C. picta* is sexually dimorphic as observed in *Trachemys scripta* turtles (TSD) but this dimorphism was masked by expression in the AK in previous studies (Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a,b, 2010b). These

results will be discussed below along with the review of the transcriptional patterns observed in other vertebrates (Fig. 3). It should be noted that while studies thus far have found that significant differential expression using AKGs in TSD reptiles reflected differences in gonadal expression (Ramsey and Crews, 2007; Shoemaker et al., 2007b; this study, Fig. 2), the possibility exists that differential AK expression may override monomorphic expression patterns in the gonads alone during gene expression quantification (Pieau and Dorizzi, 2004; Ramsey and Crews, 2007; Shoemaker et al., 2007b), and thus, interpretations involving different tissues should be treated with caution.

Adaptive TSD and sex ratio evolution require the existence of genetic variability in populations. Individual variation in gene expression was observed in our study and was larger for some genes (e.g., *Sox9*) than others (e.g., *Aromatase*) (Fig. 2), suggesting that natural selection might operate more strongly on some elements of this regulatory network than on others. The adaptive evolution of transcriptional regulation would require that this individual variation has a heritable component as seen in human and other organisms (Ayroles et al., 2009; Hodgins-

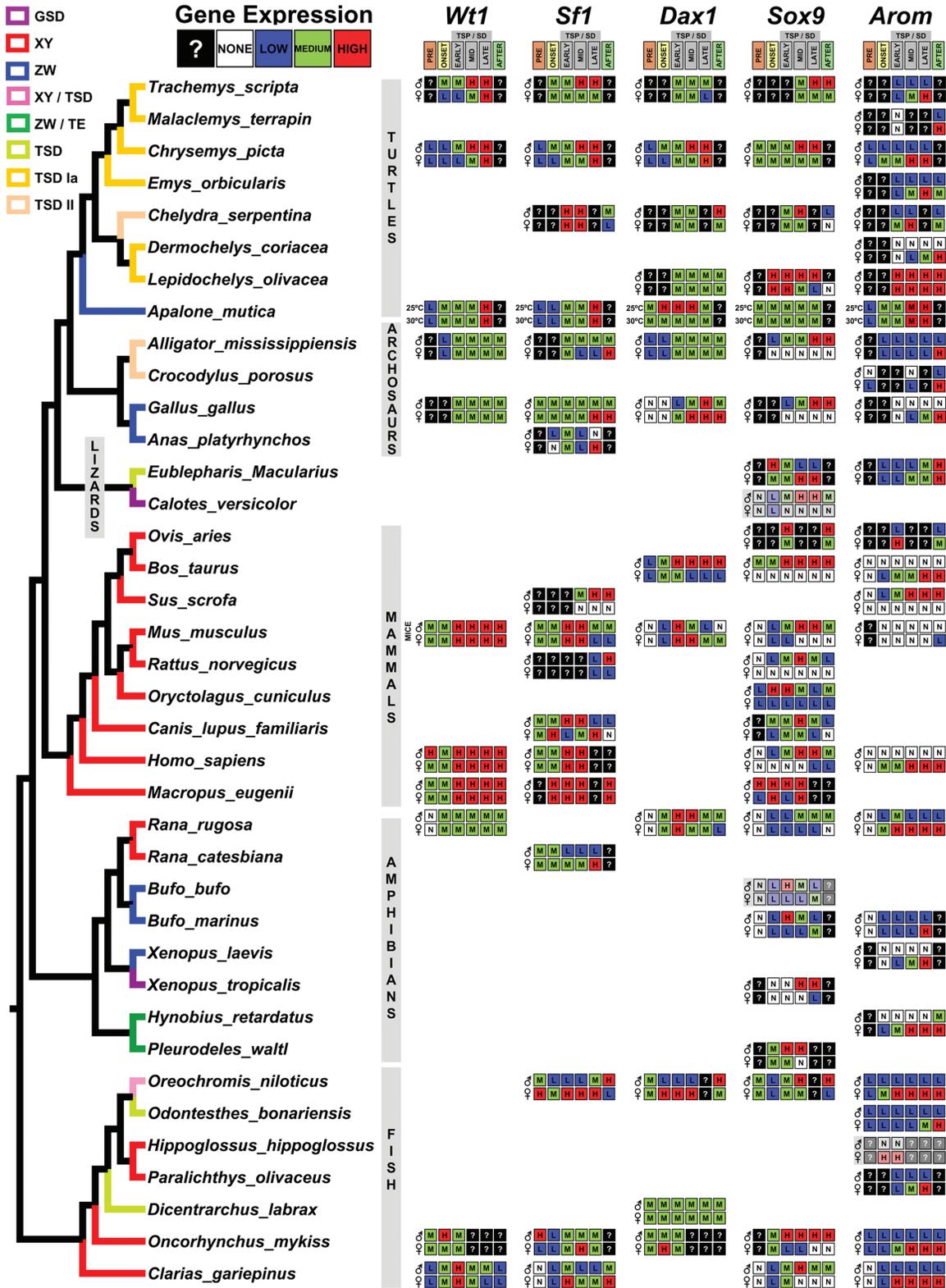


Fig. 3. Schematic transcriptional patterns of *Wt1*, *Sf1*, *Dax1*, *Sox9*, and *Aromatase* during embryonic development in a phylogenetic context for vertebrates with contrasting sex determining mechanisms. Levels of expression are color coded as not expressed (white), low (blue), medium (green), high (red), and black for unexamined stages as indicated. Stages correspond to those preceding, at the onset of, during, and after the thermosensitive period (TSP) for TSD taxa or sex differentiation (SD) for GSD taxa. Expression patterns correspond to those of male or female GSD embryos, at male-producing and female-producing temperatures in TSD taxa, and at low (25°C) and high (30°C) temperatures for the GSD *Apalone mutica* soft-shell turtles. Expression panels shaded in gray are only putative gonadal patterns. Data derive from references cited in the text. ZW / TE = species with ZZ/ZW sex chromosomes whose sex ratio is susceptible to temperature effects (GSD + EE as per Valenzuela et al., 2003).

TABLE 1. Clutch Effects on Gene Expression Detected in *Chrysemys picta* Per Gene by Stage Combination^a

Gene	Stage	12	15	17	19	19	22	22
Gene	Tissue	AKG	AKG	AKG	AK	G	AK	G
<i>Wt1</i>	DF	26	14	6	22	20	16	16
	F	1.920462	1.828173	0.267441	0.89658	0.667459	1.907696	1.621953
	P	0.037301	0.093776	0.920517	0.603016	0.775684	0.115629	0.184518
<i>Sfl</i>	DF	25	12	10	25	20	15	14
	F	1.505801	1.584029	1.460163	0.677759	0.305528	1.131046	0.099274
	P	0.13662	0.16571	0.473676	0.797543	0.979194	0.420886	0.999927
<i>Dax1</i>	DF	26	12	10	25	16	16	13
	F	0.87571	5.04906	1.294077	0.643098	3.19712	5.276564	1.607356
	P	0.632302	0.000621	0.464752	0.826019	0.134772	0.001606	0.201721
<i>Sox9</i>	DF	25	14	6	22	20	16	16
	F	1.139675	9.949656	0.577535	0.685739	0.63409	0.848521	1.669068
	P	0.357967	0.000001	0.740469	0.789383	0.800548	0.62708	0.170722
<i>Arom</i>	DF	21	13	8	21	10	14	12
	F	1.349467	1.230577	0.546811	1.837223	0.125483	1.75358	0.871299
	P	0.224095	0.32299	0.780002	0.294956	0.981932	0.199927	0.595355

^aDF, degrees of freedom; P, probability; AKG, adrenal-kidney-gonad complex; AK, adrenal-kidney; G, gonad; F, F value; *Arom*, *Aromatase*. Significant *P* values are denoted in bold.

Davis and Townsend, 2009; Skelly et al., 2009; Price et al., 2011). Our incubation experimental design followed an incomplete randomized block design, which allowed us to test for clutch effects using a factorial ANOVA, and revealed significant clutch effects at some temperature-by-stage-by-gene combinations in *Wt1*, *Dax1*, and *Sox9* (Table 1). This result suggests that there may be significant heritability *sensu lato* for gene expression, although our data preclude a strict analysis of heritability, which was not the original purpose of our experimental design. If true and this broad-sense heritability were demonstrated to have an additive genetic component, it would be the first example of heritability of gene expression related to sexual development in a TSD reptile. Such heritable variation in gene expression would represent the raw material needed for natural selection to drive the evolution of the transcriptional regulation underlying sex determination. Furthermore, the temperature-by-stage combinations that showed significant clutch effects varied, indicating that the level of broad-sense heritability among clutches might vary as well.

Wt1, the Wilms' tumor suppressor gene involved in human sex determi-

nation, encodes a transcription factor necessary during early development for the establishment of the bipotential gonad and later for the induction of *Sry* and testis development, and the maintenance of Sertoli cells and seminiferous tubules as the mammalian testis develops (Kent et al., 1995; Spotila and Hall, 1998; Hammes et al., 2001; Roberts, 2005; Gao et al., 2006; Wainwright and Wilhelm, 2010). *Wt1* produces several splice variants, two of which are conserved across vertebrates and differ by whether they contain or lack the tripeptide KTS between zinc-fingers 3 and 4 (Hammes et al., 2001). In mouse and human, these two isoforms have distinct (but not fully exclusive) functions (–KTS in the formation of the bipotential gonad and +KTS in inducing *Sry* and the male pathway) and their ratio is important for normal testicular development (Hammes et al., 2001; Du et al., 2002; Wagner et al., 2003; Gao et al., 2006; Miyamoto et al., 2008; Bradford et al., 2009; Wainwright and Wilhelm, 2010). In the marsupial tammar wallaby (*Macropus eugenii*), *Wt1* transcription is monomorphic in the developing testis and ovaries (Pask et al., 2007), and so is protein expression in embryonic gonads of mice and human (Hanley et al., 1999; Hammes et al., 2001), although levels are higher in

males after gonadal differentiation consistent with an essential role of *Wt1* for testicular maintenance. In contrast, *Wt1* transcription is higher in whole male bovine embryos, but it is uncertain whether this reflects higher gonadal expression in males (Bermejo-Alvarez et al., 2011). Orthologs of *Wt1* that play similar roles during urogenital development have been identified in non-mammalian vertebrates (Kent et al., 1995; Semba et al., 1996; Spotila and Hall, 1998; Spotila et al., 1998; Yamamura et al., 2005). Concordant with a role in testicular formation, the expression of both *Wt1* splice variants is higher at temperatures that produce males in TSD turtles such as *T. scripta* (Spotila and Hall, 1998; Spotila et al., 1998), and *C. picta* (this study, Fig. 2). However, *Wt1* expression is monomorphic in embryonic chicken (Smith et al., 1999) and *Alligator mississippiensis* gonads (Western et al., 2000). In *Rana rugosa* frogs, both isoforms are expressed at similar levels in developing testis and ovaries (Yamamura et al., 2005), while in two teleost fish (*Oncorhynchus mykiss* trout and *Clarias gariepinus* catfish) *Wt1* expression is higher in male embryonic gonads or whole male embryos than in females (Hale et al., 2011; Raghuvveer et al., 2011). Data on *Wt1* remain scarce to make strong

inferences. Given the phylogenetic relationships among vertebrates (Fig. 3) and the available data, we hypothesize that male-biased *Wt1* expression might be the ancestral vertebrate condition while monomorphic expression may have arisen at the split of tetrapods. If this were true, monomorphic *Wt1* expression would be basal in mammals and archosaurs (crocodilians and birds) while higher expression in males would be derived in turtles. The complete characterization of the action of the -KTS and +KTS isoforms in ovarian and testicular formation in non-mammalian vertebrates that exhibit sexually monomorphic levels of transcription is needed to rule out the possibility that the differential action of these splice variants is sex-specific as in mouse and human. If this were demonstrated, it would indicate that *Wt1*'s putative ancestral role in testicular formation may have been retained all along vertebrate evolution but masked in some non-mammalian lineages by the expression of alternative splice-variants.

Sf1, the steroidogenic factor 1 (also known as AD4BP and NR5A1), a gene that encodes a nuclear orphan receptor, is required for the formation of mammalian primary steroidogenic organs (adrenal gland and gonad), and for the expression of steroidogenic enzymes therein (Parker and Schimmer, 1997; Morohashi, 1999). *Sf1* expression differs between the sexes in GSD vertebrates and in contrasting ways. Indeed, *Sf1* expression is higher during male gonadal development in rat (Hatano et al., 1994), mouse (Ikeda et al., 1994), pig (Pilon et al., 1998), and trout (Hale et al., 2011) (but see Vizziano et al., 2007), while it is monomorphic in human (Hanley et al., 1999), dog (Meyers-Wallen, 2005), and wallaby (Whitworth et al., 2001). In contrast, *Sf1* expression is higher during female development in chicken (Smith et al., 1999; Yamamoto et al., 2003), although higher expression was detected in embryonic chicken testis at early developmental stages (Yamamoto et al., 2003). Expression in embryonic duck gonads appears to be more variable (Koba et al., 2008) but somewhat similar to the early male-biased and later female-biased

expression observed in chicken (Smith et al., 1999; Yamamoto et al., 2003). *Sf1* expression is also higher during female development in *Rana catesbiana* frogs (Mayer et al., 2002), *Oreochromis niloticus* tilapia (Ijiri et al., 2008), and *C. gariepinus* catfish (Raghuveer et al., 2011). Expression patterns also vary among TSD taxa. *Sf1* expression is higher at male-producing temperatures in *T. scripta* and *C. picta* TSD turtles (Fleming et al., 1999; Crews et al., 2001; this study, Fig. 2), whereas it is higher at female-producing temperatures in alligator (Western et al., 2000). In snapping turtles (*Chelydra serpentina*) no differences in expression were detected between a male-producing temperature and a pulse-temperature treatment that produces females (Rhen et al., 2007) but it is unclear if the discrepancy with other TSD turtles might be due to differences in the incubation conditions between studies or if they reflect a divergent expression pattern in *C. serpentina*. The combined data indicate that *Sf1* expression is highly labile throughout vertebrate evolution, showing patterns consistent with a role in testicular development, ovarian development, or a non-sex-specific role in general gonadal formation across and within different lineages. Given that multiple patterns are present in fish and amphibians, we hypothesize that that polymorphic *Sf1* expression is basal to vertebrates and that this polymorphism has been retained, elaborated, or lost differentially among lineages during vertebrate evolution (Fig. 3). However, further sampling is needed before conclusive statements can be made.

Dax1 is the dosage-sensitive sex-reversal [DSS], adrenal hypoplasia congenital [AHC] critical region on the X chromosome gene 1 in humans, a gene that encodes a nuclear orphan receptor (McCabe, 2007). *Dax1* was first linked to ovarian formation in mammals (Ramkissoo and Goodfellow, 1996). However, *Dax1* has been recently shown to be important for mammalian testicular determination (Meeks et al., 2003). This apparent dual role of *Dax1* is related to dosage-dependent action in eutherian mammals given its X-linkage. Namely, while the expression of a single dose

of *Dax1* in XY males (male-biased expression) is crucial for testis formation, the expression of a double dose of *Dax1* in XX females (female-biased expression) suppresses the male pathway and leads to ovarian formation (Meeks et al., 2003). Thus, overall *Dax1* expression is higher in the female mouse- and cow-developing ovary than in males (Swain et al., 1996; Ross et al., 2009). Consistent with these observations in mammals, *Dax1* expression is higher in developing chicken female and thus postulated as important in ovarian formation in birds (Smith et al., 2000; Yamamoto et al., 2003). Expression is also higher in the developing ovaries of trout (Hale et al., 2011) and tilapia (Ijiri et al., 2008). On the other hand, *Dax1* gonadal expression is sexually monomorphic in several TSD taxa including alligator (Western et al., 2000), *T. scripta* turtle (Shoemaker et al., 2007a), *C. serpentina* turtle (Rhen et al., 2007), *L. olivacea* sea turtle (Torres-Maldonado et al., 2002), *Dicentrarchus labrax* sea bass (Martins et al., 2007), suggestive of a general non-sex-specific role in gonadal formation in these vertebrates. Interestingly, temperatures that induce female-to-male reversal in tilapia (an XX/XY GSD species) also induce higher *Dax1* expression in XX males (D'Cotta et al., 2008), revealing a dual role of *Dax1* in gonadal formation in this species as it has in mammals. In contrast, *Dax1* expression in the TSD *C. picta* turtle is significantly male-biased in the gonad during the TSP (Fig. 2), indicating that it may play a more important sex-specific role in testicular formation in this species. Similarly, *Dax1* is expressed at higher levels in embryonic testis of *R. rugosa* frogs (Sugita et al., 2001; Nakamura, 2009). Given that *Dax1* expression data are relatively scarce, we tentatively hypothesize that *Dax1*'s dual role in ovarian and testicular formation might be basal in vertebrates as seen in fish (Fig. 3). Furthermore, given that *Dax1* is X-linked in eutherian mammals but it is autosomal in wallaby (Pask and Renfree, 2001), chicken, tilapia (Shirak et al., 2006; Cnaani et al., 2008; Eshel et al., 2012), and TSD taxa (which lack sex chromosomes), its dosage-sensitivity appears to be unique to eutherian

mammals and likely derived as a consequence of the transposition of the ancestral autosomal region containing *Dax1* to the eutherian X chromosome (Pask et al., 1997).

Sox9 is a member of a large family of *Sox* genes containing a *Sry*-like high-mobility group (HMG) box, and encodes a transcription factor related to testis differentiation in mammals (Morrish and Sinclair, 2002). In therian mammals, *Sox9* is a downstream target of *Sry*, and along with *Fgf9*, tips the balance towards male fate by outcompeting the female-promoting signal from *Wnt1/Rspo1* (DiNapoli and Capel, 2008) in the bipotential gonad. *Sox9* plays an important role in the differentiation of Sertoli cells, the epididymis, and müllerian ducts (Kent et al., 1996; Arango and Lovell-Badge, 1999; Vidal et al., 2001; Clarkson and Harley, 2002) and both its induction and maintenance are essential for normal mammalian testicular formation (reviewed in Wainwright and Wilhelm, 2010). As in human (Ostrer, 2001), mouse (Kent et al., 1996), pig (Parma et al., 1999), rat (Frojdman et al., 2000), dog (Meyers-Wallen, 2003), cow (Ross et al., 2009), sheep (Torley et al., 2011), rabbit (Diaz-Hernandez et al., 2008), and wallaby (Pask et al., 2002; Pask and Renfree, 2010), *Sox9* expression is higher in developing testis in chicken (Kent et al., 1996; Morrish and Sinclair, 2002; Yamamoto et al., 2003), putatively in *Calotes versicolor* lizard (Choudhary et al., 2000) (GSD: Harlow, 2004), and at male-producing temperatures in alligator (Western et al., 1999), *T. scripta* turtle (Spotila et al., 1998), *L. olivacea* sea turtle (Moreno-Mendoza et al., 1999; Torres-Maldonado et al., 2002), *C. serpentina* turtle (Rhen et al., 2007), and *C. picta* turtle (this study). Despite the similarity in the general pattern of male-biased *Sox9* expression in TSD taxa, differences in the timing of the onset of this differential transcription are observed (Fig. 3). For instance, in *C. picta*, *Sox9* is upregulated at male-producing temperatures starting at the onset of the TSP (stage 15), and as in *T. scripta*, this upregulation is accentuated from stage 17 through stage 22 (this study Fig. 2; Ramsey and Crews, 2007). This pattern contrasts with that seen in sea turtles (*L.*

olivacea) where *Sox9* expression is high in both sexes during the TSP and downregulated at female-producing temperatures after the TSP (Torres-Maldonado et al., 2001). In notable contrast to these TSD reptiles, *Sox9* expression is higher at female- than at male-producing temperatures in the TSD leopard gecko *Eublepharis macularius* (Valleley et al., 2001). This completely reversed transcriptional pattern of *Sox9* in *E. macularius* may reflect a novel rewiring of this regulatory network or the co-option of another network module during the evolution of TSD in this lineage. Supporting this hypothesis, in amphibians, *Sox9* transcription is higher in the developing testis of *R. rugosa* frogs (Takase et al., 2000) (and masked in gonad/mesonephros complexes; Nakamura, 2009), the newt *Pleurodeles waltl* (Dumont et al., 2011), the toad *Bufo marinus* (Abramyan et al., 2009), and putatively in *B. bufo* (Roco et al., 2008). Furthermore, male-specific expression is observed in *Xenopus tropicalis* frogs, also consistent with a conserved role in testicular development (El Jamil et al., 2008). However, the *Sox9* pattern in *E. macularius* is not unique among vertebrates. In fish, *Sox9* transcription is higher in female tilapia embryos at very early stages (Eshel et al., 2012), although transcription is higher later in the developing testis (Ijiri et al., 2008) as in other vertebrates. In medaka (*Oryzias latipes*), *Sox9* is not detected in either embryonic male or female gonads, while it is expressed in adult ovaries but not testis (Yokoi et al., 2002). However, *Sox9* is higher in male trout embryos (Baron and Guiguen, 2003) and developing testis in catfish (Raghuveer et al., 2011). The contrasting *Sox9* expression in medaka may reflect the earlier action of the sex-determining gene in this species, *DMY/Dmrt1Yb* (Tanaka et al., 2008), which may bypass the need of *Sox9* action during early testicular formation. Thus, it appears that *Sox9* expression is polymorphic in fish, but that this polymorphism emerges from derived patterns in teleosts from a basal condition of male-biased expression and a role in testicular development that is conserved in other fish and tetrapods, with the only known

exception of the TSD leopard gecko. Thus, divergence among tetrapods in *Sox9* is seen mostly in the timing of expression (heterochronic changes) (Fig. 3). Given the consistent male-biased *Sox9* expression in all other tetrapods, additional data in leopard gecko and other squamates are needed to test whether the pattern in *E. macularius* is indeed an evolutionary outlier. The postulated involvement of *Sox9* in some aspects of female gonadal development in amphibians (El Jamil et al., 2008; Nakamura, 2009), and the presence of two copies of *Sox9* in fish, one expressed in the testis and one in the ovary (Chiang et al., 2001; Raghuveer et al., 2011), lend support to the notion that the *Sox9* expression pattern in the leopard gecko may not be a technical artifact but reflects its recruitment for ovarian formation in this lineage.

Aromatase is a gene encoding for the enzyme responsible for the aromatization of androgens to estrogens, which plays an important role in several estrogen-dependent processes including sex differentiation (Hughes et al., 1999; Conley and Hinshelwood, 2001; Place and Lance, 2004). *Aromatase* was first thought to have arisen de novo during vertebrate evolution, but recent data revealed a more ancient origin and its possible involvement in sex steroid production in invertebrates (Castro et al., 2005; Mizuta and Kubokawa, 2007). The involvement of *Aromatase* in sex differentiation varies among vertebrates. *Aromatase* participates in late stages of gonadogenesis in the developing mouse (Greco and Payne, 1994) such that mammalian female ovarian formation has been considered mostly *Aromatase* independent (Place and Lance, 2004; Liu et al., 2010). However, comparative analysis of *Aromatase* expression paints a different picture. *Aromatase* activity or transcription is detected early in ovarian differentiation in human (George and Wilson, 1978), sheep (Torley et al., 2011) and cow (Ross et al., 2009). Counter to other mammals, *Aromatase* expression is found in the developing testis of the embryonic pig but not in ovaries (Parma et al., 1999). In contrast to the mouse but more similar to other mammals,

Aromatase is expressed in the developing chicken ovary at the onset of gonadal differentiation and thus is postulated to be more intimately involved in ovarian formation in birds (Smith and Sinclair, 2001; Vaillant et al., 2001; Yamamoto et al., 2003; Smith, 2010). *Aromatase* activity or transcription is higher at female-producing temperatures during the thermosensitive period in TSD taxa such as the turtles *Emys orbicularis* (Desvages and Pieau, 1992), *Malaclemys terrapin* (Jeyasuria et al., 1994), *C. serpentina* (Rhen et al., 2007), *T. scripta* (Ramsey and Crews, 2007), *Dermochelys coriacea* (Desvages et al., 1993), and *C. picta* (this study, Fig. 2), and after the thermosensitive period in crocodylians [alligator (Smith et al., 1995; Gabriel et al., 2001; Place et al., 2001) and *Crocodylus porosus* (Smith and Joss, 1994)], while it is high but monomorphic in *L. olivacea* (Salame-Mendez et al., 1998). Consistently, in amphibians *Aromatase* expression is higher in the developing ovary of the *Hynobius retardatus* salamander (Sakata et al., 2005), *B. marinus* toad (Abramyan et al., 2009), *R. rugosa* frog, and *Xenopus* frog (Miyashita et al., 2000). In fish, *Aromatase* expression is also higher in the developing ovaries of the GSD tilapia (D'Cotta et al., 2001; Ijiri et al., 2008), trout (Vizziano et al., 2007; Hale et al., 2011), catfish (Raghuveer et al., 2011), as well as in the TSD *Odontesthes bonariensis* pejerrey (Fernandino et al., 2008) and *Paralichthys olivaceus* flounder (Kitano et al., 1999). Likewise, *Aromatase* transcription is higher in whole female larvae of *Hippoglossus hippoglossus* halibut (van Nes et al., 2005) but it is uncertain if this reflects higher gonadal expression in females. Taken together, these data confirm that similar to *Sox9*, high lability exists in the timing of the onset of dimorphic *Aromatase* expression in TSD taxa (Fig. 3) (Valenzuela and Shikano, 2007) despite a conserved female-biased expression pattern with the only exception of *L. olivacea* sea turtles that exhibit monomorphic *Aromatase* activity. Heterochronic changes are not exclusive to reptiles but extend to other vertebrates and likely reflect the divergence in how central is *Aroma-*

tase for ovarian development. For instance, collectively, these studies suggest that as in mouse, early gonadal development is not *Aromatase*-dependent in TSD species and much less so in crocodylians and *L. olivacea*. Yet, the observations that the dimorphic *Aromatase* expression starts at early stages of sex determination (Yoshimoto et al., 2008), and precedes gonadal differentiation in catfish (Raghuveer et al., 2011) argue in favor of an ancestral role of *Aromatase* at the early stages of vertebrate ovarian development that was taken over by other elements in this regulatory network during tetrapod evolution.

PERSPECTIVES

The data reviewed here and the novel data from *C. picta* from this study demonstrate that patterns of expression have diverged among TSD lineages, sometimes among families as in the case of *Sox9* (e.g., *C. picta* and *T. scripta*— Emydidae, vs. *L. olivacea* Cheloniidae; Testudinae vs. Squamata), sometimes among Orders as for *Sox9*, *Sf1*, and *Wt1* (e.g., Testudines vs. Squamata vs. Crocodylia), and even within families (e.g., Emydidae) as for *Dax1*. These observations strengthen the notion that TSD encompasses more than a single mechanism from a developmental perspective (Valenzuela and Shikano, 2007; Valenzuela, 2008a). Significant divergence is also observed in the presence/absence or directionality of dimorphic expression as well as timing within and among GSD and TSD lineages. Such divergence of the gene regulatory network underlying sex determination and differentiation would represent an example of developmental systems drift (True and Haag, 2001), in direct contrast with the consistent gene expression across species expected if sex-determining systems (TSD or GSD) were pleiomorphic and developmentally conserved (Valenzuela, 2008a). Further research is warranted to fully unravel the mystery of the evolution of sex determination in vertebrates and the following are some areas that may prove particularly insightful.

The rapid increase of high-throughput technologies available today provide a unique opportunity to better understand the composition of the network underlying sexual development, both for model and non-model systems, given that our knowledge of the full set of elements involved in this process remains incomplete even for humans. Particularly intriguing is the study of the involvement of non-coding RNA in sex determination as recently found in humans (McFarlane and Wilhelm, 2009), and the general influence of other epigenetic processes in sexual development. Additionally, experimental biology remains a fundamental approach that is essential to decipher the regulation of the sexual development network and to test for the existence of network modules that might be differently affected by natural selection. In this regard also, comparative transcriptomics and the use of a phylogenetic framework will shed light on how this network has evolved for over 300 million years of vertebrate evolution and what might be its evolutionary potential.

EXPERIMENTAL PROCEDURES

Phylogenetic Hypothesis

Phylogenetic relationships among turtles were based on a recent turtle supertree of all recognized living chelonians (Iverson et al., 2007) pruned as described in Valenzuela and Adams (2011). Phylogenetic relationships among all other amniotes follow Organ et al., (2009), with modification according to Crawford et al., (2012) to reflect the monophyly of Archosaurs (crocodylians and birds) and which places turtles as sister to Archosaurs to the exclusion of Lepidosaurs (Tuatara, Lizards, and Snakes). Phylogenetic relationships among Amphibians Pyron and Wiens, (2011) and then among fish follow Li et al., (2008) and Orti et al., (unpublished data).

Gene Expression

Freshly laid eggs from multiple nests were distributed uniformly and randomly among programmable incubators (Valenzuela, 2008a) set at 26°C (female-producing), 31°C (male-producing). Eggs were incubated in boxes ³/₄ filled with play sand set at a

TABLE 2. Number of Samples of *Chrysemys picta* Included in the Analysis of Gene Expression^a

Stage	Tissue	T°	Gene				
			<i>Wt1</i>	<i>Sf1</i>	<i>Dax1</i>	<i>Sox9</i>	<i>Arom</i>
12	AKG	26°C	30	29	30	29	24
12	AKG	31°C	32	30	29	31	28
15	AKG	26°C	19	17	17	19	19
15	AKG	31°C	21	20	18	21	18
17	AKG	26°C	4	4	4	4	1
17	AKG	31°C	7	10	11	7	12
19	G	26°C	13	13	9	13	1
19	G	31°C	16	15	13	16	12
19	AK	26°C	18	19	18	18	12
19	AK	31°C	19	19	20	19	15
22	G	26°C	16	14	14	16	9
22	G	31°C	16	13	14	16	15
22	AK	26°C	16	16	16	16	16
22	AK	31°C	16	13	16	16	9

^aT°, incubation treatment; *Arom*, *Aromatase*; AKG, adrenal-kidney-gonad complex; AK, adrenal-kidney; G, gonad.

4% water content (v/w) (Valenzuela, 2009). Humidity was maintained by replacing lost water (as determined by weight loss) before egg sampling or weekly otherwise, and boxes were rotated daily within incubators following a clock-wise and front-to-back schedule. A target of up to 20–30 embryos was collected per stage and temperature. Final sample sizes are listed in Table 2. Embryos were stored in RNeasy[®] at –20°C for later use. RNA was extracted from the adrenal-kidney-gonad complex (AKG) of stage 12–17, and from the gonad and AK separately from stages 19–22, using Qiagen's (Valencia, CA) RNeasy Kits (Valenzuela, 2008a). RNA was quantified with a NanoDrop[®] ND-1000 Spectrophotometer, and its quality assessed by the presence of ribosomal bands in agarose gels. Total RNA was reverse-transcribed with (dT)₂₀ primers using Superscript VILO (Invitrogen, Carlsbad, CA). Individual samples were kept separate and analyzed without pooling. Gene expression was assessed by real-time qPCR using Brilliant[®] SYBR[®] Green qPCR Master Mix in an Mx3000P thermocycler (Stratagene, La Jolla, CA) using *C. picta*-specific primers (Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a,b, 2010b). Standard curves were generated from

pure miniprep cloned DNA of a fragment of the cDNA of interest serially diluted and run in duplicate in each qPCR to ensure technical repeatability of the results as in previous studies (Valenzuela et al., 2006; Valenzuela and Shikano, 2007; Valenzuela, 2008a,b, 2010b). qPCR conditions included: 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 30 sec, 60°C for 1 min, 72°C for 1 min; and a standard dissociation-curve cycle of 95°C for 1 min, 55°C for 30 sec, and ramping up towards 95°C with readings taken every 0.5°C (79 readings), followed by 95°C for 30 sec. Dissociation profiles were inspected to confirm that a single cDNA fragment was amplified. Statistical outliers and samples that amplified more than a single product as determined from dissociation profiles were excluded from further analysis. Final sample sizes are presented in Table 2 per gene, stage, and treatment. Log₂-transformed expression obtained by qPCR normalized to the housekeeping gene β -actin was compared across stages and treatments using ANOVA (Valenzuela, 2008a) in R software version 2.11.1.

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