

## Review

# Analyzing the coordinated gene network underlying temperature-dependent sex determination in reptiles<sup>☆</sup>

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## ABSTRACT

Although gonadogenesis has been extensively studied in vertebrates with genetic sex determination, investigations at the molecular level in nontraditional model organisms with temperature-dependent sex determination are relatively new areas of research. Results show that while the key players of the molecular network underlying gonad development appear to be retained, their functions range from conserved to novel roles. In this review, we summarize experiments investigating candidate molecular players underlying temperature-dependent sex determination. We discuss some of the problems encountered unraveling this network, pose potential solutions, and suggest rewarding future directions of research.

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**Abbreviations:** MPT, male-producing temperature; FPT, female-producing temperature; TSD, temperature-dependent sex determination; GSD, genotypic sex determination; ESD, environmental sex determination; TSP, temperature-sensitive period; AKG, adrenal-kidney-gonad; GAM, gonad-adrenal-mesonephros; MIS, Müllerian-inhibiting substance; DMRT1, doublesex mab3 related transcription factor 1; SRY, sex-determining region of the Y chromosome; SOX9, SRY-like HMG-box containing transcription factor 9; SOX8, SRY-like HMG-box containing transcription factor 8; FOXL2, forkhead box protein L2; WNT4, wingless integration site family member 4; DAX1, DSS-AHC critical region on the X chromosome; PP1, protein phosphatase type I; SF1, Steroidogenic factor 1; WT1, Wilms tumor 1; FGF9, fibroblast growth factor 9; RSP01, R-spondin 1; RT-PCR, reverse transcriptase polymerase chain reaction; qPCR, quantitative real-time PCR; ISH, *in situ* hybridization.

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## 1. Introduction

Vertebrates exhibit a dazzling array of sex-determining mechanisms that can generally be classified as belonging to one of two categories: genotypic sex determination (GSD) and environmental sex determination (ESD). Gonochoristic vertebrate species exhibiting genotypic sex determination utilize a heritable genetic element that initially directs the gonad down one of two sexual trajectories. In contrast, the sex-determining factor in organisms with ESD is derived from the physical or biotic environment [1,2]. Crocodylians, tuataras, many turtles and some lizard species exhibit a particular mode of ESD known as temperature-dependent sex determination (TSD), in which the incubation temperature of the developing embryo directs sexual fate. First documented by Charnier [3], species with TSD are sensitive to the action of temperature during a specific window of development known as the temperature-sensitive period (TSP) (for review see [1]). It is thought that species with TSD commonly lack heteromorphic sex chromosomes, although there is evidence of the co-existence of GSD and TSD mechanisms within particular lizard species [4], as occurs in some fish [5,6].

Downstream of the action of either a genetic or an environmental sex-determining trigger lies a complex network of molecular interactions and cellular behaviors leading to the formation of an ovary or a testis. This has best been studied in mammals and birds, lending much insight into both the process of gonadogenesis in GSD organisms as well as points of therapeutic intervention in a variety of human sexual development disorders (for review see [7–11]). As information concerning the genetic players of GSD has blossomed, investigators have become curious if these networks are conserved in other types of sex-determining mechanisms. Studies investigating the molecules underlying TSD began appearing in the mid-1990s and have since become a field of their own (for review see [12–14]). It is an important line of research because a comparative approach informs our understanding of the evolution of signaling pathways, molecular function and interactions and cellular behaviors across diverse taxa.

It is useful to emphasize that all development occurs within a contextual environment, be it at the level of neighboring cells, adjacent tissues, or external surroundings. Organisms with TSD offer a unique system in which to study the interplay between environment and development. Simply by shifting the incubation temperature of the egg during the temperature-sensitive period, one can re-set embryonic gonadogenesis. Full sex-reversal is caused by changing the embryonic environment, and this ability offers an opportunity to study many aspects of organogenesis including cell migration, fate choice, differentiation, and cell–cell signaling.

### 1.1. Species with TSD: who to study?

The use of nontraditional model systems is both a blessing and a curse: we choose to study them for their insights into the similarities and differences between taxa, but this often results in new stumbling blocks. Working with non-model critters presents technological challenges, and experimental techniques must be re-optimized for each particular species. Further, for many TSD species, seasonal breeding and relatively slow rates of development inherently means that experiments must often be optimized and completed over several seasons. Not only does this draw out the time needed for conclusive results to be gathered, it also begets complexity in the interpretation of data collected from multiple clutches, mothers, months within a season, and even years. Thus, it becomes important to ask which organisms initially make the most sense to utilize in studying the genetics underlying TSD.

Species with TSD are highly variable in their patterns of temperature-sensitivity. For many turtle species, low incubation temperatures during development lead to male embryos (male-producing temperature or MPT), whereas higher incubation temperatures lead to females (female-producing temperature or FPT) ([15–18], for review see [19]). In contrast, crocodiles and some turtle and lizard species exhibit a different mode of TSD in which extreme cool and warm temperatures produce females, while intermediate temperatures lead to males (for review see [19–21]). Studies in the laboratory of either pattern reveals that incubation at transitional or fluctuating temperatures produces clutches of eggs with varying sex ratios, and intersex embryos are uncommon.







While some conservation in the function of molecules involved in gonadogenesis exists across sex-determining mechanisms [22], it is also likely that some divergence has occurred as well. This is particularly true in terms of the initial triggers underlying sex determination. Investigating the temperature-sensing mechanism may unearth different molecular explanations for different TSD patterns. Further, divergence within molecular function may extend to the downstream network involved in sex determination and commitment, and possibly even within gonad differentiation.

The species currently being utilized in studies investigating the genetics of TSD are listed in Table 1, along with particular details of their TSD patterns. While we do not advocate the elimination of any particular species as unworthy of study, we do suggest that perhaps our efforts would best be concentrated on species chosen for particular criteria. These might include species that are most amenable to laboratory study due to size and availability, have previously garnered the most data, or are predicted to be most evolutionarily informative. The selection of a few key species to focus on would mean a more rapid accumulation of data and a clearer understanding of the TSD molecular network. It must be noted that understanding other aspects of TSD, including ecological, behavioral and evolutionary questions (for review see [23]), clearly do not fall within the same parameters as molecular studies, and should and do cover a much broader range of taxa.

### 1.2. A common vernacular

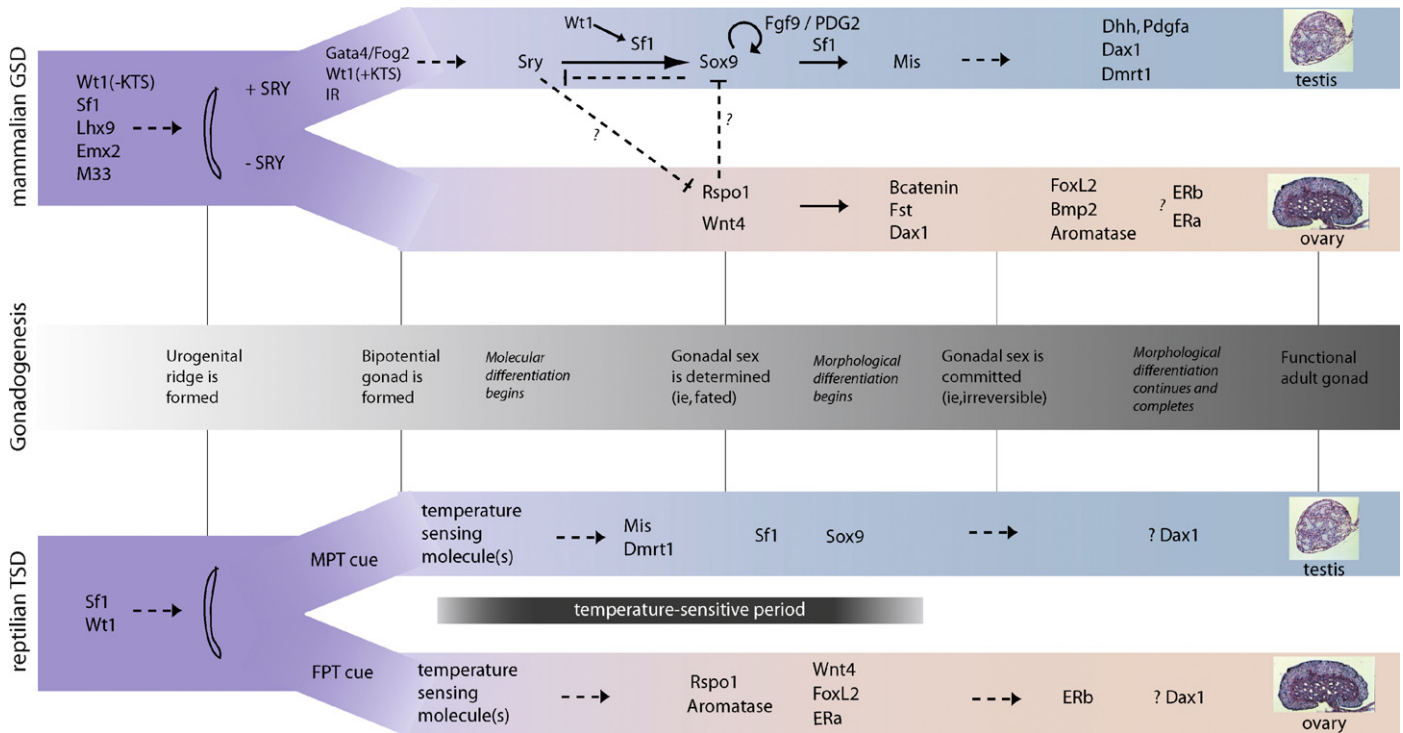
In the deceptively simple statement “sex is determined” lies a range of meaning. Plausible interpretations include that the cells of the gonad have begun expressing sex-specific markers, that some individual embryos at this point are no longer sex-reversible, or that all embryos at this point are no longer sex-reversible. This confusion is problematic, and thus a possible way of distinguishing between critical events in the timeline of gonadogenesis in organisms with TSD is presented in Fig. 1 (text contained in center gray bar). Initially, during the formation of the urogenital ridge and the bipotential gonad, individuals of TSD species are equipotent in their ability to become either sex. Temperature acts on this bipotential gonad, perhaps cumulatively, via target temperature-sensing molecule(s), and thus molecular differentiation begins as the temperature-sensitive period opens. Molecular differentiation continues as sex-specific pathways begin to be expressed, directing the gonad towards its eventual sexual fate. Sex becomes “determined”, or fated, such that if nothing perturbs the system, the gonad will continue in the developmental trajectory it has begun. At this point, gonads at different temperatures are no longer equal, but the TSP window remains open and sex is still reversible. Next, morphological differentiation begins as cells adopt different fates and reorganize, forming new, sex-specific structures within the gonad. Thus, molecular differentiation precedes morphological differentiation. Subsequently, the TSP window closes, at which point sex is “committed” and is irreversible. Sex-specific morphological

**Table 1**  
Organisms historically used in studies examining the molecular network underlying temperature-dependent sex determination.

Organism	Pattern of TSD	Temperature to produce a single-sex clutch <sup>a</sup>	Critical stages in embryonic gonadal development					Staging and gonadogenesis references	Molecular studies references	
			Egg lay	Genital ridge formation	Bi potential gonad formation	TSP (in developmental stages) <sup>b</sup>	Onset of morphological differentiation			Hatch
Red-eared slider turtle ( <i>Trachemys scripta</i> )		MPT (26 °C) FPT (31 °C)	0		by 15	15–20 15–19	18/19	26	[17,125,126]	[41,42,43,44,54,57,58,77,51,89,93,98,99,114,123]
American alligator ( <i>Alligator mississippiensis</i> )		MPT (32.5–33 °C) FPT (30 or 35 °C)	0	12/13	by st 20	21–24	21/22 22/23	28?	[20,127–130]	[47,55,76,91,92]
Olive Ridley sea turtle ( <i>Lepidochelys olivacea</i> )		MPT (26–27.5 °C) FPT (32–33 °C)	0	12–13	21–23	20–23 24–27 (Days 20–27 for both temps)	26/27	31	[18,131–133]	[85,86,87,88]
Painted turtle ( <i>Chrysemys picta</i> )		MPT (25 °C) FPT (30.5 °C)	0			16–22		26	[16,126,141]	[45,56,111]
Snapping turtle ( <i>Chelydra serpentina</i> )		MPT (23–27 °C, 99%) FPT (20 °C, >29.5 °C)	0			14–19 14–16, 14–19		26	[126,134–137]	[46]
Leopard gecko ( <i>Eublepharis macularius</i> )		MPT (31–33 °C, 90%) FPT (23–28 or 34 °C)	30			32–37		40	[1,138–140]	[90]

<sup>a</sup> These temperatures are thought to produce 100% of the given sex under laboratory conditions, except where indicated.

<sup>b</sup> Temperature-sensitive period is defined as the period during which sex can be reversed in some individuals when a group of eggs are temperature-shifted.



**Fig. 1.** Genetic events underlying gonad determination and differentiation in GSD mammals and TSD reptiles. A model of the molecular network underlying mammalian genetic sex determination is given in comparison to species with TSD. It is important to note that within the model, gene names in the mammalian network represent known dimorphic function, while in the reptilian network represent sex-specific gene expression. Solid lines imply direct regulatory relationships while dashed lines imply indirect or undescribed relationships. Some parts of the model have been shown conclusively, while others are supported by evidence. Developmental time proceeds from left to right. Stages of gonad development are described in the middle of the figure (gray box), and lines extending from this box attempt to align important gonad events across sex and sex-determining mechanism. It is notable that other GSD species, such as chick, may have divergent timing of events and function of various factors. The mammalian GSD network was selected to provide a framework against which to compare TSD studies, and for other GSD species, the reader is referred to other recent reviews (see [12,13]).

differentiation continues and eventually results in a fully formed gonad.

While deviations from this generalized timeline exist, such as in *Lepidochelys olivacea* where the TSP window closes at MPT before morphological differentiation is observable [18], it provides a framework for discussion. An important distinction arises from this theoretical framework: most notably in organisms with TSD, sex determination does not equal final commitment to a sex. As studies investigating the molecular network underlying TSD progress and gene function are elucidated, scientists must take care when specifying particular steps of the pathway.

## 2. Analysis of candidate genes putatively involved in temperature-dependent sex determination

The development of a vertebrate gonad can be thought of in two phases: the initial construction of an organ from which a variety of outcomes are possible, and the subsequent narrowing of these possibilities into one particular fate. Specifically, this translates to (a) the formation of an equipotent urogenital ridge and its successor, the bipotential gonad, and (b) the molecular and morphological differentiation that determines gonadal sex and results in a fully formed testis or ovary. The analysis of the molecular network underlying TSD that follows is broken down into these processes, with candidate genes being placed within a certain phase or sex-specific trajectory based on information from both GSD and TSD species. However, because the actual role(s) of these gene products in TSD remains unclear, their placement within these categories is merely parsimonious, and certainly will not prove to be correct in all cases. The level of convergence that exists across phyla in the function of these molecular players remains to be determined.

As previously described, various species have been used in molecular studies of TSD, and the timing of the TSP window is different across species. In the discussion that follows, particular stages are detailed so that comparisons across species can be made. The reader is referred to Table 1 for further clarification, and to place these stages within the various steps of gonad development. True species-specific differences in expression patterns are found in some of the following studies. However, it is likely that differences in the techniques utilized to assay expression, the tissues used, the varying staging criteria and the actual stages examined also contribute to variation seen within the data. Interpreting the differences in these results continues to be a challenge in piecing together the molecular network underlying TSD.

### 2.1. Formation of the bipotential gonad

In mammals, the formation of the urogenital ridge and subsequent bipotential gonad from underlying mesonephric and coelomic epithelial tissues utilizes several factors, including *Sf1*, *Wt1*, *Emx2*, *Lhx9*, and *M33* (for review see [8,11]). Only two of these, *Sf1* and *Wt1*, have been examined thus far in organisms with TSD. *Steroidogenic factor 1* (*Sf1*, also known as *Ad4BP* and *Nr5a1*) is an orphan nuclear receptor that regulates transcription of many downstream genes, including several steroidogenic enzymes (for review see [24]). In early gonadogenesis, it appears to modulate cell proliferation and prevent apoptosis in the forming bipotential gonad in both sexes [25]. It also plays several later testis-specific roles: in concert with *Sry* (*Sex-determining region of the Y chromosome*) it appears to upregulate a specific enhancer element of *Sox9* (*Sry-like HMG-box containing transcription factor 9*) [26], and in con-

junction with *Sox9*, it directly upregulates *Mis* (Müllerian-inhibiting substance) [27–30].

*Wilms tumor 1* (*Wt1*), a nuclear zinc-finger transcription factor, is also expressed throughout the mammalian urogenital ridge in both sexes and appears to prevent apoptosis in the forming bipotential gonad [31–34]. Two spliceoforms of *Wt1*, distinguishable by only three amino acids, play different roles in gonadogenesis: *Wt1(+KTS)* appears to lead to the initial upregulation of *Sry* [35,36], while *Wt1(-KTS)* may directly upregulate *Sf1* [37–39]. Thus, in mammals, *Sf1* and *Wt1* both play an early non-sex-specific function in bipotential gonad formation as well as later testis-specific roles, and are good candidates to examine for function across sex-determining mechanisms.

### 2.1.1. *Sf1*

*Sf1* expression in the developing gonad of organisms with TSD has been examined in three species of turtle and the American alligator. In all cases, *Sf1* transcripts are detected throughout the bipotential gonad in the earliest stages examined, as well as during the TSP and differentiation [40–46]. This expression may indicate that a function for *Sf1* in the formation of the gonad has been retained across sex-determining mechanisms.

However, quantification of transcript levels between the sexes gives differing results across species. In the red-eared slider turtle, *Trachemys scripta*, significantly greater expression at MPT occurs in isolated gonadal tissue throughout gonadogenesis (stages 15–23) [41–43]. In contrast, expression in the painted turtle, *Chrysemys picta*, in combined adrenal-kidney-gonad (AKG) complexes is monomorphic at all stages examined (stages 9–22) except at one stage prior to the TSP (stage 12) during which expression is higher at MPT [44]. This generally monomorphic expression pattern may be due to the well-described masking effect that occurs when steroidogenic adrenal tissue, known to express *Sf1* in mouse [47,48], is included in the expression analysis of the AKG (see [49–51]). In *Chelydra serpentina*, the snapping turtle, expression levels are monomorphic between MPT gonads and MPT → FPT shifted gonads (FPT gonads were not studied), except at one stage (5 days post-shift), where *Sf1* interestingly rose in response to FPT [45]. Similarly, in the American alligator, *Alligator mississippiensis*, levels of expression appear qualitatively higher in FPT gonads at the close of the TSP (stages 23–25) [46]. While some of these differences may be due to true species-specific differences in *Sf1* expression, some are likely confounds of the techniques and tissues utilized.

Localization of *Sf1* transcript in the cells of the *T. scripta* gonad has been described by *in situ* hybridization. *Sf1* mRNA is initially spread throughout the bipotential gonad early in the TSP at both MPT and FPT (stage 15); at MPT, it subsequently becomes localized first in circular structures, possibly surrounding germ cells (stage 17), and then in testis-specific sex cords (stages 19, 23) [43]. At FPT, *Sf1* expression decreases and becomes more diffuse throughout the medullary region as development progresses (stages 17–23). Furthermore, shifting eggs from MPT → FPT or FPT → MPT during the TSP causes a decrease or increase, respectively, in gonadal levels of *Sf1* expression, as well as the expected change in the localization of transcripts within the developing gonad [42,43]. Effects of estrogen and aromatase inhibitor treatment on *Sf1* expression were also investigated in the slider turtle and are discussed in depth in another review in this issue [43,52].

Differing results from these studies in the timing of sex-specific *Sf1* expression leaves room for interpretation of the possible roles of *Sf1* in organisms with TSD. What is clear is that *Sf1* initially is expressed strongly in the bipotential gonad of several species, consistent with a conserved role in cell proliferation or apoptosis in early gonad formation, similar to mammalian function. Subsequently, in at least two turtle species, its expression is

temperature-responsive and may correlate to a function in the determination or development of the testis, similar to its several testis-specific roles in mouse.

### 2.1.2. *Wt1*

The two alternatively spliced isoforms of *Wt1* important in mouse gonadogenesis also exist in both slider turtle [53] and alligator [54], although differential expression or function of each remains uninvestigated. In three TSD species, *Wt1* expression is detectable throughout gonadogenesis in both sexes, but the relative levels of *Wt1* observed between the sexes differs across these studies. In *C. picta*, AKG complexes analyzed by qPCR reveal expression of *Wt1* throughout development (stages 9–22) [55]. This expression is monomorphic except at one stage prior to the TSP where MPT expression exceeds FPT (stage 12) [55]. In the American alligator, RT-PCR analysis of AKGs (stages 20–23) and in isolated gonads (stages 24–27) reveals *Wt1* expression is similar between the sexes and appears to increase throughout development [46].

In *T. scripta*, Northern blot analysis confirms the presence of *Wt1* transcripts in AKG complexes at both MPT and FPT throughout gonadogenesis as well (stages 14–20), although relative levels are not clear due to variation in loading as evidenced by fluctuation in actin band strength [53,56]. In an elegant protein analysis by immunocytochemistry, WT1 was localized in *T. scripta* gonads in somatic cell nuclei in the primitive sex cords that occur in both MPT and FPT gonads during the temperature-sensitive period (stages 16–18), and later during differentiation in testis-specific sex cords and ovary-specific cortical cells (stages 20–23) [57]. Further, *Wt1* protein appeared to be correlated to patterns of cell proliferation, further supporting a conserved role with mammalian function [57].

To summarize, studies of both *Sf1* and *Wt1* indicate that transcripts are present throughout gonadogenesis at both MPT and FPT, consistent with an early role in bipotential gonad formation at both temperatures. Later male-specific function is also a possibility that remains to be investigated. It has been proposed that the concurrently dimorphic expression (MPT > FPT) of both *Sf1* and *Wt1* observed in *C. picta* AKG complexes prior to the TSP (stage 12) may indicate a role for these gene products in opening or shaping the window of thermosensitivity [44,55]. The search for the temperature-sensing molecule(s) that initially sends the bipotential gonad towards a testicular or ovarian fate has proved difficult, and these hypotheses will continue to be tested.

## 2.2. Testis-determining pathway

The formation of a testis from its bipotential gonad precursor is a complex process involving many molecular and cellular interactions that has been intensely studied in vertebrates with GSD (see [7,8,11,58]). Five particular genes involved in testis determination and differentiation in mammals have also been studied in organisms with TSD, and are discussed here, namely *Sox9*, *Sox8*, *Fgf9*, *Mis* and *Dmrt1*.

Excitingly, the search for the direct target of mammalian sex-determining factor *Sry* has finally been solved. A long-standing postulation has been recently verified by Sekido and Lovell-Badge, namely that *Sry*, in concert with *Sf1*, binds to a promoter element of the gene *Sox9* [26]. *Sox9* is in the same family of HMG-box containing transcription factors as *Sry*, and is also a male-determining factor both necessary and sufficient for mammalian testis formation. Loss of *Sox9* causes male-to-female sex reversal in both humans [59,60] and mice [61,62], while transgenic XX mice containing a copy of *Sox9* develop testes [63,64]. The closely related *Sox8* appears to reinforce the function of *Sox9*, but is not able to replace it [61]. While *Sry/Sf1* action initiates pre-Sertoli cell *Sox9* expression, *Fibroblast growth factor 9* (*Fgf9*) is required to maintain

its expression and is thought to contribute to Sertoli cell differentiation [65–67]. Loss of *Fgf9* leads to male-to-female sex reversal in some individuals, and it has been shown to play an antagonistic role together with *Sox9* in apposition to the *Wnt4* signaling pathway [67]. Subsequently, the coordinated action of *Sox9* and *Sf1* directly upregulates *Mis* (also known as *Anti-Müllerian hormone*, *Amh*) [29,30]. *Mis* is secreted by Sertoli cells in the developing testis and, via the specific receptor *MisRII*, causes the degradation of the Müllerian ducts, Anlagen that otherwise develop into the uterus, cervix and fallopian tubes in mammalian females [68,69].

After sex has been determined in the mammalian testis, *Dmrt1* (*Doublesex mab3 related transcription factor 1*) function is critical to vertebrate testis differentiation [70,71]. It is one of the few genes found to have homologs involved in sex differentiation across widely diverse taxa, including *Drosophila*, *C. elegans*, and vertebrates, including human, mouse, turtles, birds, alligator, fish and lizards [72–79]. While it appears to play a more downstream role in testis differentiation in mammals [80], it has been proposed to be a master sex-determining gene (i.e., a non-mammalian equivalent of SRY) in both chicken (*Gallus gallus*) and medaka (*Oryzias latipes*) [77,78,81,82], although further studies are needed to confirm this role (e.g., [83]).

### 2.2.1. *Sox9*, *Sox8* and *Fgf9*

The ability of *Sox9* to “replace” the action of *Sry* in mammals made it an obvious candidate for a critical role upstream in testis development in organisms with TSD as well. Studies in three species of turtles and the leopard gecko show similar *Sox9* expression patterns: expression is detectable in both sexes early in gonad development and then becomes restricted to the developing testis during the end of the temperature-sensitive period. In slight contrast, expression in the alligator gonad is undetectable in either sex early in development, but similarly becomes upregulated in the developing testis at the end of the TSP.

An elegant set of studies in the sea turtle, *L. olivacea*, demonstrates the expression pattern and temperature-response of *Sox9* in the developing gonad [84–86]. In this species, the TSP lasts for 8 days during development, corresponding to stages 20–23 at MPT and to stages 24–27 at FPT [18]. Expression analyses by RT-PCR demonstrate *Sox9* is present early in developing gonads at both MPT and FPT (stages 23–25) and is later retained at MPT but is undetectable at FPT (stages 26, 27) [86,87]. Embryos incubated at MPT and then shifted to FPT for further incubation reveal that by 12 days post-shift, expression of *Sox9* responds to the new incubation temperature and is downregulated [86]. *Sox9* protein is detected in a similar pattern; early in development, *Sox9* is found in primitive sex cords in both MPT and FPT gonads (stages 22, 24) and is then restricted to MPT (stage 26) [84]. Furthermore, when gonads are grown *in vivo* or cultured *in vitro* and exposed to sex-reversing temperature shifts in either direction (MPT → FPT and FPT → MPT), *Sox9* protein levels change in the expected direction in response (decrease and increase, respectively) [85].

The data observed in *L. olivacea* is consistent with data from three other species with TSD. In *T. scripta*, initial expression analyses of AKG complexes showed monomorphic expression in both sexes throughout gonadogenesis (stages 15, 17, 20) [53,76]. However, subsequent studies revealed that this lack of sex-specificity is likely due to the inclusion of the strong *Sox9* expression observed in dorsal metanephric tissue present in the AKG [50]. qPCR analysis on isolated gonads reveals *Sox9* transcripts at both MPT and FPT early in the TSP (stages 16–17), followed by significantly higher expression of *Sox9* at MPT at the close of the TSP and through differentiation (stages 19–23, Fig. 2A) [88]. *In situ* hybridization localizes *Sox9* in the MPT gonad to preSertoli cells in developing sex cords (Fig. 2B) [50]. In *C. serpentina*, *Sox9* is expressed at MPT throughout

gonadogenesis and is downregulated in response to a shift to FPT [45]. Similarly, in the leopard gecko, *Eublepharis macularius*, *Sox9* is detected by whole mount *in situ* hybridization during the TSP in both MPT and FPT gonads (stage 36) and is then restricted to MPT gonads at the end of the TSP (stages 37, 40) [89].

In contrast, *Sox9* transcripts in the American alligator are not detected by RT-PCR in either sex early in the TSP (stages 20–23); expression is upregulated at MPT at the end of the TSP and during testis differentiation (stages 24–27) [90]. Coincidentally, the tissue sources used within this study varied; early in development the gonad is unable to be removed cleanly from underlying tissue and therefore gonad-adrenal-mesonephros (GAM, equivalent to AKG) must be analyzed (stages 20–23), while isolated gonad is possible later in development (stages 24–27). At MPT, *Sox9* transcripts initially localize to faint expression in scattered cells (stages 23.5, 24), and subsequently to strong expression in developing sex cords (stage 25) [90,91].

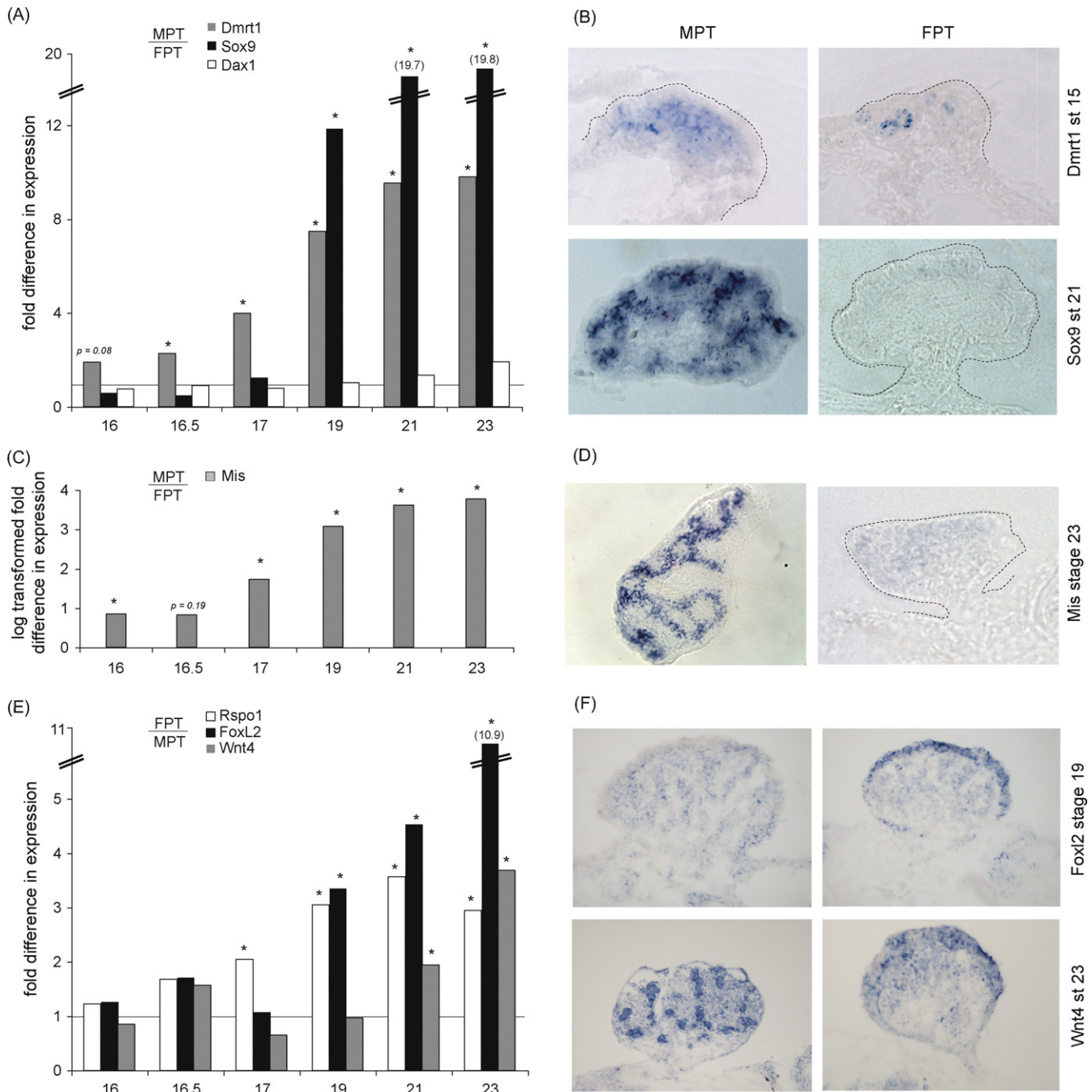
In summary, these data suggest that *Sox9* is not involved in the initial steps of sex determination, as is seen in mammals. However, its function may be critical for final commitment to a testicular fate. As described above, gonadal sex in organisms with TSD remains plastic until the end of the temperature-sensitive period. Dimorphic expression of genes involved in commitment to a sexual fate would not be necessary in the developing gonad until the end of the TSP when the window of sex-reversibility is closing. From this perspective, the data from all examined species with TSD (five) are consistent with the hypothesis that *Sox9* plays a role in the commitment of the bipotential gonad to a testicular fate.

Both *Sox8* and *Fgf9* expression have been examined in one TSD turtle species each. *Sox8* was cloned and investigated in *T. scripta* [92], while *Fgf9* was examined in *C. serpentina* [45]. Both genes are expressed at similar levels at both MPT and FPT throughout gonadogenesis, and future investigation may reveal their roles, if any, in gonadal development in organisms with TSD.

### 2.2.2. *Mis*

In organisms with TSD, *Mis* has been best studied in *T. scripta*. Expression analysis of both pooled AKG complexes and isolated gonads reveals higher expression at MPT than FPT early in the bipotential gonad and throughout gonadogenesis (stages 16–23) (Fig. 2C) [88,92]. *In situ* hybridization localizes transcripts to somatic cells within developing testicular sex cords (Fig. 2D) [50]. Further, *Mis* expression is rapidly downregulated in gonads shifted from MPT to FPT early in the TSP, suggesting the repression of a testis-specific function [88]. To our knowledge, the only other TSD organism in which *Mis* has been examined is the American alligator. In this species, dimorphic expression also appears early in gonad development in a few medullary cells at MPT and continues to increase throughout the TSP (stages 22–25), with no detectable specific expression at FPT [91].

Thus, gonadal mRNA expression of *Mis* in the alligator and turtle becomes upregulated in putative males prior to upregulation of *Sox9*, similar to the case in chicken [93,94]. This suggests a divergence from the relationship seen in mammals in which *Sox9* initially upregulates *Mis* expression. In turtles, early *Sox9* expression is detectable in both sexes from the beginning of the TSP. If a role for *Sox9* in the early stages of sex determination in the turtle testis exists, it must be regulated at the protein level as is the case in mammals. In both mouse and human, *Sox9* is localized to the nuclei of preSertoli cells in the testis, exerting transcriptional control over downstream genes, while in ovarian cells remains cytoplasmic and thus inactive as a transcription factor [95,96]. Future work should investigate whether a similar nuclear-cytoplasmic shuttling system is important in temperature-dependent sex determination in the turtle. However, what seems more likely from the data thus far is the



**Fig. 2.** Gonadal expression of various candidate sex-determining genes in the slider turtle, *Trachemys scripta*. (A, C, E) Expression data is reproduced from qPCR study in which each value is the average of  $n = 3$  samples, where one sample represents expression in a pooled group of 20–30 gonads from 10 to 20 individual turtle embryos (data redrawn from [88,122]). Asterisks indicate statically significant difference *within gene within stage between MPT and FPT* at the  $\alpha = 0.05$  level. Gene of interest expression was normalized to *PP1*, a house-keeping gene whose expression does not change with sex or stage. X-axis represents developmental stage [125]. Y-axis is either: (A and C) fold difference in expression between MPT and FPT, or (B) log-transformed fold difference in expression between MPT and FPT. For example, stage 23 *FoxL2* expression at FPT is nearly 11 times MPT expression, while bars below 1.0 have greater expression in gonad of the denominator sex. (C) Because expression of *Mis* at FPT is so low as to reach the limit of detectability (essentially equal to zero), fold expression values at MPT become inappropriately inflated and are therefore plotted as log-transformed data. (B, D, F) Whole mount *in situ* hybridization localizes gene expression patterns within turtle gonads from embryos at various stages (data from [50,88]).

exciting possibility that *Sox9* plays a role in the final commitment of a gonad to a testicular fate.

### 2.2.3. *Dmrt1*

In three species with TSD, male-specific expression of *Dmrt1* is observed either early or late in the temperature-sensitive period, but its functional role remains unclear. In the American alligator,

expression is initially detectable in urogenital ridges at both MPT and FPT (stages 20–22), and subsequently becomes higher at MPT during the end of the TSP (stages 23–25) [75]. In *L. olivacea*, expression was not examined early in the male TSP. It is initially detectable in both sexes (stages 23–25); after the TSP, expression increases at MPT (stages 25–31) and becomes undetectable at FPT (stages 26–31) [87]. In *T. scripta*, expression during the TSP in both AGK

complexes and isolated gonad tissue is significantly greater at MPT and low or undetectable at FPT (stages 15–26) (Fig. 2A) [76,88,97]. *In situ* hybridization reveals cellular localization of *Dmrt1* expression to somatic cells in developing testicular sex cords (Fig. 2B) [50].

Functional data analyzing possible involvement of *Dmrt1* in testis determination or differentiation has come in two forms. Firstly, gonadal *Dmrt1* expression is rapidly upregulated in *T. scripta* embryos shifted from FPT to MPT during the TSP, suggesting a necessary function in male development [88]. This result is supported by studies in the snapping turtle, *C. serpentina*, in which expression is attenuated in gonads shifted in the opposite direction from MPT to FPT during the TSP, consistent with a repression of *Dmrt1* function in putative female embryos [45]. Secondly, Murdock and Wibbels showed that exogenous application of estrogen to the eggshells of developing *T. scripta* embryos prior to the TSP inhibits *Dmrt1* expression for the duration of the TSP [98]. Taken together, these data strongly implicate the involvement of *Dmrt1* in testis formation and reveal a sensitivity to both temperature and estrogen, although not necessarily a direct one. *Dmrt1* still holds the captivating possibility of being a master sex-determining gene in several GSD species, and may possibly be directly regulated by temperature in TSD species.

### 2.3. Intersecting pathways

Several factors have been shown to play complex roles in the development of both the mammalian testis and the ovary, including *Dax1* and *Wnt4*. Differing functions of the same molecule can be regulated by the timing and localization of expression, the presence/absence of other co-regulators, and the presence/absence of activators/repressors of the molecules themselves. The function of the nuclear receptor *Dax1* (*Dosage-sensitive sex-reversal, Adrenal hypoplasia congenital on the X chromosome 1*, also known as *Nr0b1* and *Ahch*) is unique in that the otherwise highly conserved DNA-binding domain has been replaced in *Dax1* by a protein binding domain thought to bind other nuclear receptors (see [8]). Understanding its role in gonadogenesis in mammals has been complicated over the past decade, first being theorized as a female-specific gene [99,100] and now being understood as critical to testicular development as well [101–103]. It has been suggested to operate at multiple timepoints within both sexes as a “common” regulator of gonad development with several functions (for review see [104]).

*Wnt4* (*Wingless-type MMTV integration site family member 4*) signaling has also been implicated in the development of the mammalian reproductive system in both sexes (for review see [105]), mediating the initial formation of the Müllerian ducts and regulating the migration of steroidogenic mesonephric cells into the developing gonad [106,107]. However, it also seems to play several sex-specific roles during gonadogenesis. In the ovary, *Wnt4* acts via *Follistatin* to prevent the formation of a testis-specific coelomic blood vessel [108]. Further, antagonistic action and/or competition to reach threshold levels between *Wnt4* and *Sox9/Fgf9* may tip the balance between female and male development [67]. In the testis, gonadal expression of *Sox9*, *Mis*, *Dhh* and *Sf1* is decreased in *Wnt4*<sup>−/−</sup> XY mice and can be rescued by ectopic *Wnt4*, suggesting an active role for *Wnt4* in testicular development [109]. *Dax1* and *Wnt4* expression in organisms with TSD is discussed below.

#### 2.3.1. *Dax1*

In organisms with TSD, gonadal *Dax1* is expressed at similar levels between the sexes in four species of turtle and the American alligator. Through developmental time this expression either decreases significantly (*T. scripta*), decreases slightly and then dramatically (*L. olivacea*), increases slightly (*A. mississippiensis*, *C. picta*),

or does not change (*C. serpentina*) [45,46,87,88,110]. It remains to be seen what each of these timing-correlated changes mean at the functional level. Interestingly, expression in *C. serpentina* was correlated to clutch, and hatchling ovaries contained twice the level of *Dax1* as hatchling testes [45]. *In situ* hybridization reveals localization of *Dax1* expression in developing sex cords at both MPT and FPT, in the cortex of FPT gonads, and in cells of both the Müllerian and Wolffian ducts [88].

The sexually monomorphic expression pattern of *Dax1* in organisms with TSD is no surprise, as roles for *Dax1* exist in both the developing testis and ovary of mammals. Further analysis of the function of *Dax1* in TSD gonads is warranted, and due to the complexity observed in the role of *Dax1* in mammalian systems, these studies may prove more complicated than unraveling other parts of the TSD network.

#### 2.3.2. *Wnt4*

In organisms with TSD, *Wnt4* has only been examined in the red-eared slider turtle, *T. scripta*. Analysis of isolated gonad tissue reveals similar expression levels at MPT and FPT during the TSP (stages 16–19), and a subsequent significant increase at FPT over MPT during ovarian differentiation (stages 21, 23) (Fig. 2E) [88]. *In situ* hybridization localizes this expression across the gonad of both sexes, with increased concentration of transcript in sex cords at MPT, in the cortical region at FPT, and in developing Müllerian ducts (Fig. 2F) [88]. Similar to *Sox9*, if *Wnt4* plays a role in the commitment of the bipotential gonad to a sexual fate (rather than the earlier step of sex determination), its expression should be dimorphic near the close of the TSP. Indeed, our data are consistent with a possible role of this signaling molecule in governing ovarian development at the level of commitment to a sexual fate.

### 2.4. Ovary-determining pathway

The pathway underlying mammalian testicular determination and differentiation has been elucidated more clearly and quickly than that underlying ovarian development, and unfortunately this has been mimicked in studies of TSD as well. The critical roles of estrogen, its receptors, ER $\alpha$  and ER $\beta$ , and *aromatase* in the development of the ovary in organisms with TSD are thoroughly discussed by Ramsey and Crews in this issue [52]. Here, we discuss two genes involved in ovarian development in both GSD and TSD vertebrates, *FoxL2* and the recently implicated *Rspo1*.

The transcription factor *Forkhead box protein L2* (*FoxL2*) was first linked to gonad development by associations between *FoxL2* point mutations and BPES syndrome, symptoms of which include gonadal dysgenesis and premature ovarian failure in humans [111,112]. *FoxL2* is expressed in the developing ovary of mouse and chick embryos [113], and is involved in postnatal mammalian granulosa cell differentiation [114]. Recent studies have shown that double *FoxL2/Wnt4* knockout mice exhibit female-to-male sex reversal, and have identified *FoxL2* promoter elements that may be mechanistically responsible for this reversal [115,116].

The most recently discovered player in ovarian development is *R-spondin1* (*Rspo1*), which, excitingly, is the first identified single gene to cause full female-to-male sex reversal when mutated in humans [117]. It is a signaling ligand that can regulate Wnt and B-catenin signaling pathways [118,119]. It appears that *Rspo1*, via activation of B-catenin, and reinforced by *Wnt4* signaling, is required for mammalian ovary determination [120,121]. The discovery of this putatively sex-determining gene is a breakthrough in unraveling the intricacies of the signaling and transcription network that occurs in an XX gonad. It has thus been proposed that *Rspo1* reinforces *Wnt4* in its antagonism with *Sox9/Fgf9*, and a threshold amount of either within the bipotential gonad tips the



balance towards one developmental trajectory over the other, thus committing the bipotential organ to a sexual fate [7].

#### 2.4.1. *FoxL2*

*FoxL2* was first described in a TSD organism in *T. scripta*, where expression is present in AKG complexes at both temperatures early in the TSP (stage 15) and later becomes higher at FPT than at MPT (stages 17, 20) [113]. Subsequent analysis by qPCR on isolated gonads gave slightly different results: expression is similar at MPT and FPT early in the TSP (stages 16–17), and becomes significantly greater at FPT during the end of the TSP (stages 19–23) (Fig. 2E) [88]. *In situ* hybridization further localized *FoxL2* transcript to cells in the developing ovarian cortex (Fig. 2F) [88]. Similarly, in the snapping turtle, *C. serpentina*, gonadal expression of *FoxL2* was found to increase in response to a shift from MPT to FPT during the TSP, indicating temperature-sensitivity and suggesting a role for *FoxL2* in ovarian development [45].

#### 2.4.2. *Rspo1*

The latest addition to the set of sex determination players was also recently examined in *T. scripta*. In isolated gonad tissue, expression is significantly higher at FPT than at MPT early in the TSP and continues to become increasingly disparate between the sexes (stages 17–23) (Fig. 2E) [122]. Further, when embryos are shifted from FPT to MPT early in the TSP, expression of *Rspo1* in the gonad is rapidly downregulated, consistent with the hypothesis that repression of an ovarian-specific function is necessary for testicular development [122]. This exciting result constitutes one of the earliest significant dimorphisms in expression level of an ovary-specific factor in organisms with TSD. It implicates a role for *Rspo1* in ovarian development, as well as a responsiveness to temperature, and merits further investigation of *Rspo1* as a critical candidate ovarian sex-determining gene.

### 3. Conclusion

As have been detailed above, descriptive studies analyzing mRNA expression patterns have been the most practically feasible place to begin investigating the involvement of candidate genes in temperature-dependent sex determination. Clearly, however, these studies can only take our understanding so far. We would do well to heed a lesson from *Drosophila* sex determination: presence of transcript does not necessarily mean a functional transcript, that the transcript is spliced consistently in different cellular environments, or that protein spliceforms are functionally equivalent. Thus, interpretation of expression data, while worthwhile and critical to laying the foundation of future studies, must all be taken with a proverbial grain of salt.

What is required next are analyses of protein expression, localization, and most importantly, function, if we are to truly understand the hierarchical cascade of gene action underlying TSD, and any evolutionary relationship of these actions with GSD. Experiments that move analyses in nontraditional model systems away from descriptive studies and into functional analyses have already begun. In their exciting gonad explant organ culture studies, Merchant-Larios and colleagues have provided us with a system just waiting for the application of techniques used commonly in model systems to assess function [85]. Overexpression and knockdown techniques can be optimized for the application to *in vitro* gonads, opening the door to understanding both the sufficiency and necessity of these molecules in the development of the temperature-determined gonad. Further, both the Capel and Kuratani laboratories have undertaken studies examining cell–cell interactions and cellular behaviors using explant chimeras of turtle/mouse or turtle/chick tissues [123,124]. These

creative applications of techniques used routinely in model systems must continue if we are to actually understand the roles of known candidate molecules or, most excitingly, uncover the direct targets of temperature in the developing gonad.

Understanding expression data from studies of organisms with TSD within a broader context has resulted in a discussion of the level of conservation and divergence in molecular expression and function, cellular behaviors, and tissue development across sex-determining mechanisms. Unfortunately, we cannot fully assess the relative importance of conserved or novel roles of common molecular players until we have better techniques to elucidate their function. For certain there will be some conservation and some divergence as we expand studies of the molecular mechanisms of sex determination into nontraditional model systems. How much of each and what meaning we can derive from them remains to be seen. Regardless, these studies lend insight into the evolution of molecular action and, at a broader level, the evolution of developmental pathways.

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